10088571.062201 MAR 2002

FORM PTO-1390
(REV. 11-94)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

ATTORNEY'S DOCKET NUMBER
8969-033-999
10/088571

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/09173	September 19, 2000	September 23, 1999

TITLE OF INVENTION
SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES

APPLICANT(S) FOR DO/EO/US
Jens BUCHARDT, Niels Taekker FOGED, Morten MELDAL, Jean-Marie DELAISSE, Michael ENGSIG, Mercedes FERRERAS, Morten KARSDAL, Maria del Carmen OVEJERO, Christine Bruun SCHIODT and Bent WINDING

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (Unexecuted)

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

INTERNATIONAL APPLICATION NO. PCT/EP00/09173 10/088571	INTERNATIONAL FILING DATE September 19, 2000

17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	25 -20	5	X $18.00	$ 90.00
INDEPENDENT CLAIMS	5 -3	2	X $84.00	168.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	☐

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): **CHECK ONE BOX ONLY** ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) $710.00		
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $740.00		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1,040.00		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) $100.00		
☒ Filing with EPO or JPO search report $890.00	$	890.00
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than 20 ☒ 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).	$	130.00
0 TOTAL OF ABOVE CALCULATIONS	=	1,278.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).	- $	0.00
SUBTOTAL	=	1,278.00
Processing fee of $130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).	+	
0 TOTAL FEES ENCLOSED	$	1,278.00

a. ☐ A check in the amount of $___ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $1,278.00 to cover the above fees. A copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150. A copy of this sheet is enclosed.

18. ☐ Other instructions

n/a

19. ☒ All correspondence for this application should be mailed to

PENNIE & EDMONDS LLP
1667 K Street, N.W.
Washington, D.C. 20006

20. ☒ All telephone inquiries should be made to

Max Bachrach NAME for Paul J. Zegger 33,821	Max Bachrach SIGNATURE	45,479 REGISTRATION NUMBER	March 21, 2002 DATE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (c)) - SMALL BUSINESS CONCERN	Docket No. 46865/57841 (8969-033-999)

Serial No.	Filing Date	Patent No.	Issue Date
10/088,571	March 21, 2002		

Applicant/ Patentee: Jens BUCHARDT, ET AL.

Invention: SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES

I hereby declare that I am:

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: **Nordic Bioscience A/S**

ADDRESS OF CONCERN: **Herlev Hovedgade 207, DK-2730 Herlev, Denmark**

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above identified invention described in:

- ☐ the specification filed herewith with title as listed above.
- ☒ the application identified above.
- ☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☐ no such person, concern or organization exists.
- ☐ each such person, concern or organization is listed below.

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Klaus Eldrup-Jørgensen

TITLE OF PERSON SIGNING OTHER THAN OWNER: CEO

ADDRESS OF PERSON SIGNING: **Nordic Bioscience A/S**
Herlev Hovedgade 207
DK-2730 Herlev, Denmark

SIGNATURE: _____ DATE: Sept. 11, 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jens BUCHARDT *et al.*

Application No.: To be assigned (371 of International Application No. PCT/EP00/09173)

Group Art Unit: To be assigned

Filed: Herewith

Examiner: To be assigned

For: SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES

Attorney Docket No.: 8969-033

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

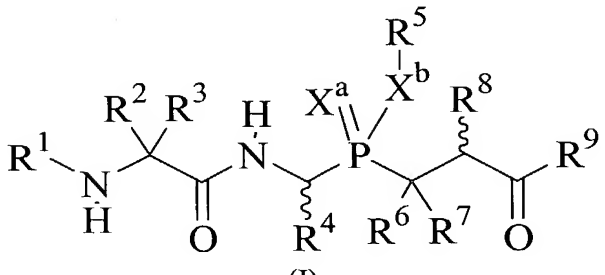
Prior to examination of the above-captioned application, please enter the following amendments and remarks into the file.

IN THE CLAIMS:

Please cancel claims 1-27 without prejudice. Please add the following new claims:

--

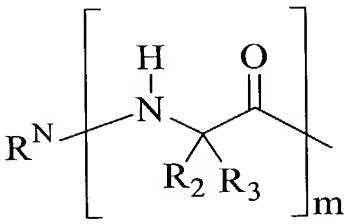
28. (New) A method of treating a metabolic bone disease, comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound of formula (I):



(I)

or a pharmaceutically acceptable salt thereof, wherein:

R^1 is hydrogen, an amino-protecting group, alkyl, alkenyl, alkynyl aryl, R^{1b}-NH-$CR^{1c}R^{1d}$-CO-, R^{1e}-CO-, or is of the formula:



R^{1b} is hydrogen, alkyl, alkenyl, alkynyl aryl, or a natural or unnatural α-amino acid or a peptide comprising the same;

each of R^{1c} and R^{1d} is independently hydrogen, a radical corresponding to a side chain of a natural and non-natural α-amino acid, alkyl, alkenyl, alkynyl, aryl, or R^{1e}-CO-;

R^{1e} is hydrogen, alkyl, alkenyl, alkynyl, aryl, or R^{1f}-SO_2-;

R^{1f} is alkyl, alkenyl, alkynyl or aryl;

R^N is an amino protecting group;

m is an integer;

each of R^2, R^3, R^4 and R^8 is independently R^{1c} or R^{2a}-CH_2;

R^{2a} is aryl or a heteroalicylic or heteroaromatic group;

R^5 is hydrogen, alkyl, alkenyl, alkynyl or aryl;

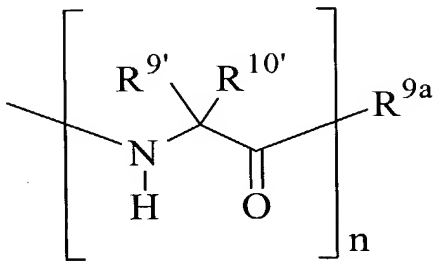
each of R^6 and R^7 is independently hydrogen or alkyl;

R^9 is R^{9a} or R^{9b};

R^{9a} is R^{3a}-X^c-;

R^{3a} is alkyl, alkenyl, alkynyl, aryl, a heteroalicylic or heteroaromatic group, or a group as defined for R^2;

R^{9b} is a group of the formula



each of $R^{9'}$ and $R^{10'}$ is independently as defined above for R^2;
n is an integer; and
each of X^a, X^b, and X^c is independently O, S, or NH.

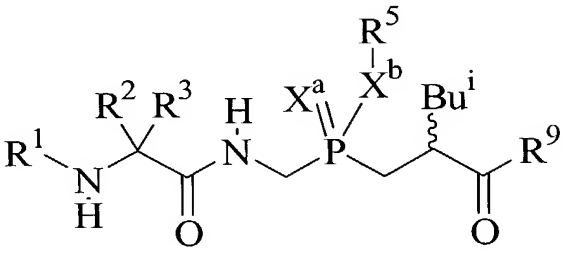
29. (New) The method of claim 28 wherein the compound of formula (I) or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a metalloproteinase.

30. (New) The method of claim 29, wherein the compound of formula (I) or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a matrix metalloproteinase.

31. (New) The method of claim 28, wherein the compound of formula (I) or a pharmaceutically acceptable salt thereof in a concentration of 50 mM or less is able to reduce significantly and by more than 50% compared to the appropriate vehicle treated control, one or more of the following activities: the osteoclast invasion in the *collagen invasion assay* or in the *bone lining cell invasion assay*, the osteoclastic pericellular collagenolysis or the distance of migration in *the pericellular collagenolysis assay*, the TGF-b induced increase in accessible surface area of a culture of bone lining cells, or the osteoclastic bone resorption induced by treatment with TGF-b of a bone lining cell layer seeded on a bone substratum, or the TGF-b induced decalcification of cultured foetal mouse calvariae in the *bone lining cell elongation assay*, the decalcification or the number of invading osteoclast in a culture of foetal mouse metatarsals in the *metatarsal assay*, the removal in calvarial cultures of demineralised collagen fibres by osteoclasts in the subosteoclastic resorption zone or by bone lining cells in the resorption pits left by the osteoclasts in *the demineralised collagenolysis assay*, or the release of ^{45}Ca in *the calvarial decalcification assay*, or in a daily dose of 100 mg/kg or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 20% compared to the appropriate vehicle treated control, one or more of the following activities: the release of ^{3}H in the *tetracycline-labelled bone resorption assay*, or the number, area or mortality rate in the *bone metastasis assay*.

32. (New) The method of claim 28 wherein the compound of formula (I) has a K_i-value of 100 nM or less with one or more of the MMPs, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 or MMP-20, and a K_i-value at least 100 times higher than the lowest observed K_i-value for an MMP with two or more of the MMPs, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14 or MMP-20.

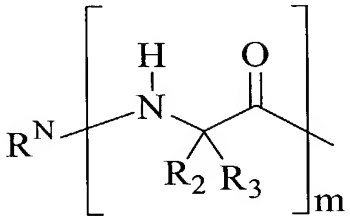
33. (New) A compound of formula (II):



(II)

or a pharmaceutically acceptable salt thereof, wherein:

R^1 is hydrogen, an amino-protecting group, alkyl, alkenyl, alkynyl aryl, R^{1b}-NH-$CR^{1c}R^{1d}$-CO-, R^{1e}-CO-, or is of the formula:



R^{1b} is hydrogen, alkyl, alkenyl, alkynyl aryl, or a natural or unnatural α-amino acid or a peptide comprising the same;

each of R^{1c} and R^{1d} is independently hydrogen, a radical corresponding to a side chain of a natural and non-natural α-amino acid, alkyl, alkenyl, alkynyl, aryl, or R^{1e}-CO-;

R^{1e} is hydrogen, alkyl, alkenyl, alkynyl, aryl, or R^{1f}-SO_2-;

R^{1f} is alkyl, alkenyl, alkynyl or aryl;

R^N is an amino protecting group;

m is an integer;

each of R^2 and R^3 is independently R^{1c} or R^{2a}-CH_2;

R^{2a} is aryl or a heteroalicylic or heteroaromatic group;

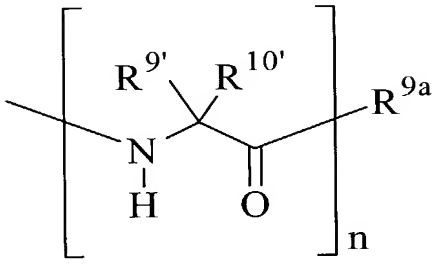
R^5 is hydrogen, alkyl, alkenyl, alkynyl or aryl;

R^9 is R^{9a} or R^{9b};

R^{9a} is R^{3a}-X^c-;

R^{3a} is alkyl, alkenyl, alkynyl, aryl, a heteroalicylic or heteroaromatic group, or a group as defined for R^2;

R^{9b} is a group of the formula



each of $R^{9'}$ and $R^{10'}$ is independently as defined above for R^2;

n is an integer; and

each of X^a, X^b, and X^c is independently O, S, or NH.

34. (New) A method of treating a metabolic bone disease, comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound of claim 33.

35. (New) The method of claim 34, wherein the compound acts by inhibition of the production or action of a metalloproteinase.

36. (New) The method of claim 35, wherein the compound acts by inhibition of the production or action of a matrix metalloproteinase.

37. (New) The compound of claim 33, wherein said compound, in a concentration of 50 mM or less, is able to reduce significantly and by more than 50% compared to the

appropriate vehicle treated control, one or more of the following activities: the osteoclast invasion in the *collagen invasion assay* or in the *bone lining cell invasion assay*, the osteoclastic pericellular collagenolysis or the distance of migration in *the pericellular collagenolysis assay*, the TGF-b induced increase in accessible surface area of a culture of bone lining cells, or the osteoclastic bone resorption induced by treatment with TGF-b of a bone lining cell layer seeded on a bone substratum, or the TGF-b induced decalcification of cultured foetal mouse calvariae in the *bone lining cell elongation assay*, the decalcification or the number of invading osteoclast in a culture of foetal mouse metatarsals in the *metatarsal assay*, the removal in calvarial cultures of demineralised collagen fibres by osteoclasts in the subosteoclastic resorption zone or by bone lining cells in the resorption pits left by the osteoclasts in *the demineralised collagenolysis assay*, or the release of ^{45}Ca in *the calvarial decalcification assay*, or in a daily dose of 100 mg/kg or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 20% compared to the appropriate vehicle treated control, one or more of the following activities: the release of ^{3}H in the *tetracycline-labelled bone resorption assay*, or the number, area or mortality rate in the *bone metastasis assay*.

38. (New) The compound of claim 33, wherein said compound has a K_i-value of 100 nM or less with one or more of the MMPs, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 or MMP-20, and a K_i-value at least 100 times higher than the lowest observed K_i-value for an MMP with two or more of the MMPs, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14 or MMP-20.

39. (New) A compound having or comprising a sequence described in Table 10:

Table 10

Phosphinate Based Peptide Derivative	P5	P4	P3	P2	G^{pc}L	2'	3'	4'	5'
01-01		V	A	Y		K	S	R	G
01-02			A	Y		K	S	G	
01-03			V	Y		R	S	G	
01-04		G	L	A		W	L	P	G
01-05			L	A		W	L	G	

Phosphinate Based Peptide Derivative	P5	P4	P3	P2	$G^{pc}L$	2'	3'	4'	5'
01-06			L	A		Q	L	G	
01-07	A	G	P	L		Y	A	R	G
02-01		L	M	Y		Y	A	P	G
02-02		I	M	Y		Y	A	P	G
02-03		K	M	Y		Y	A	P	G
02-04		Z	M	Y		Y	A	P	G
02-21		Q	M	Y		Y	A	P	G
02-05		L	L	Y		Y	A	P	G
02-06		L	I	Y		Y	A	P	G
02-07		L	P	Y		Y	A	P	G
02-08		L	M	R		Y	A	P	G
02-09		L	M	F		Y	A	P	G
02-10		L	M	Y		L	A	P	G
02-11		L	M	Y		I	A	P	G
02-12		L	M	Y		J	A	P	G
02-13		L	M	Y		Y	L	P	G
02-14		L	M	Y		Y	I	P	G
02-15		L	M	Y		Y	J	P	G
02-16		L	M	Y		Y	M	P	G
02-17		L	M	Y		Y	Y	P	G
02-22		L	M	Y		Y	W	P	G
02-18		L	M	Y		Y	A	L	G
02-19		L	M	Y		Y	A	I	G
02-20		L	M	Y		Y	A	J	G
04-01				Y		Y			
04-19				Y		Y	W		
04-20				Y		Y	M		
04-21			M	Y		Y			
04-18		I	L	F		L	M	I	G
04-03		T	L	Y		L	D	G	
04-04		V	L	Y		T	L	S	G
04-05		I	M	Y		V	K	F	G
04-07		T	L	Y		R	A	I	G
04-09		T	L	R		L	F	F	G
04-10		I	L	R		M	A	P	G
04-11		S	L	F		R	D	I	G
04-12		L	M	F		Y	L	S	G
04-13		I	M	Y		Y	M	T	G
04-14		K	F	Y		L	Y	A	G
04-15		Y	I	Y		T	M	P	G
04-16		S	M	A		Y	H	G	
04-17		I	M	R		L	S	E	G
04-02		I	L	L		N	L	I	G
04-06		L	I	E		R	K	G	
04-08		E	F	Y		K	Y	N	G
05-01		T	A	S		M	F	G	
05-02		M	Y	T		Y	K	L	G
05-03		T	R	K		S	E	L	G
05-04		T	R	Q		S	E	L	G
05-05			S	M		L	Y	A	G
05-06		L	A	A		Y	F	Y	G
05-07		E	S	N		Y	Y	G	
05-08		J	V	A		S	T	G	G
05-09		J	Y	M		L	Q	L	G
05-10		J	Y	M		L	K	L	G
05-11		V	F	K		M	A	K	G
05-12		N	R	A		F	Q	A	G

Phosphinate Based Peptide Derivative	P5	P4	P3	P2	$G^{PC}L$	2'	3'	4'	5'
05-13		R	V	S		N	Y	G	G
05-14		G	J	K		Y	N	R	G
05-15		G	J	F		E	S	L	G
05-16		V	S	H		A	T	F	G
05-17		Y	P	E		S	A	S	G
05-18		J	M	V		L	Q	F	G
05-19		H	F	K		Q	G	F	G
05-20		Q	P	H		F	Y	D	G
05-21		S	J	D		G	V	E	G
05-22		R	J	D		T	L	J	G
05-23		R	P	P		L	L	G	
05-24		K	Y	F		G	P	M	G
05-25		G	M	G		P	F	L	G
05-26		T	N	P		N	V	E	G
05-27		G	T	V		A	K	Q	G
05-28			J	L		L	F	J	G
05-29		K	T	M		V	Q	L	G
05-30		K	T	M		V	K	L	G
05-31		Y	M	R		H	S	G	
05-32		N	V	V		Y	L	E	G
05-33		D	A	H		D	F	G	
05-34		T	P	L		E	A	D	G
05-35		A	P	A		L	A	Q	G
05-36		R	P	A		Q	M	R	G
05-37		Y	A	Y		K	Y	E	G
05-38		Y	A	Y		Q	Y	E	G
05-39		T	J	E		V	A	G	
05-40		V	A	K		Q	R	G	

or a pharmaceutically acceptable salt thereof, a sequence related thereto by the substitution of one or more amino acids without decreasing the effectiveness or without decreasing the selectivity of the compound as a metalloproteinase inhibitor, or a compound acting as a molecular mimic of any such compound in interacting with a metalloproteinase, wherein $G^{PC}L$ is -GlyΨ(PO_2-CH_2)Leu-.

40. (New) A method of treating a metabolic bone disease, comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound of claim 39.

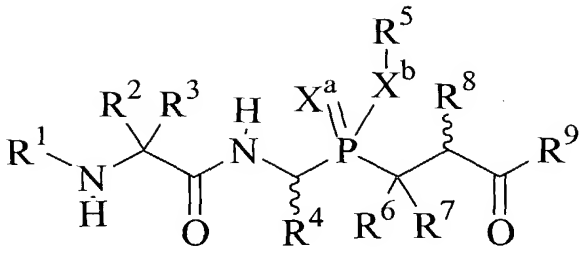
41. (New) The compound of claim 39, wherein the compound acts by inhibition of the production or action of a metalloproteinase.

42. (New) The compound of claim 41, wherein the compound acts by inhibition of the production or action of a matrix metalloproteinase.

43. (New) The compound of claim 39 wherein said compound, in a concentration of 50 mM or less, is able to reduce significantly and by more than 50% compared to the appropriate vehicle treated control, one or more of the following activities: the osteoclast invasion in the *collagen invasion assay* or in the *bone lining cell invasion assay*, the osteoclastic pericellular collagenolysis or the distance of migration in *the pericellular collagenolysis assay*, the TGF-b induced increase in accessible surface area of a culture of bone lining cells, or the osteoclastic bone resorption induced by treatment with TGF-b of a bone lining cell layer seeded on a bone substratum, or the TGF-b induced decalcification of cultured foetal mouse calvariae in the *bone lining cell elongation assay*, the decalcification or the number of invading osteoclast in a culture of foetal mouse metatarsals in the *metatarsal assay*, the removal in calvarial cultures of demineralised collagen fibres by osteoclasts in the subosteoclastic resorption zone or by bone lining cells in the resorption pits left by the osteoclasts in *the demineralised collagenolysis assay*, or the release of ^{45}Ca in *the calvarial decalcification assay*, or in a daily dose of 100 mg/kg or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 20% compared to the appropriate vehicle treated control, one or more of the following activities: the release of ^{3}H in the *tetracycline-labelled bone resorption assay*, or the number, area or mortality rate in the *bone metastasis assay*.

44. (New) The compound of claim 39, wherein said compound has a K_i-value of 100 nM or less with one or more of the MMPs, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 or MMP-20, and a K_i-value at least 100 times higher than the lowest observed K_i-value for an MMP with two or more of the MMPs, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14 or MMP-20.

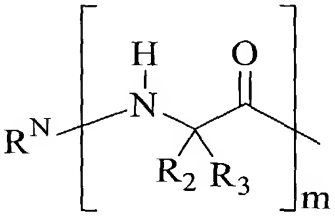
45. (New) An anti-bone resorption agent comprising compound of formula (I):



(I)

operatively linked to a ligand targeting a proteinase involved in bone resorption or to the environment of the proteinase, wherein:

R^1 is hydrogen, an amino-protecting group, alkyl, alkenyl, alkynyl aryl, R^{1b}-NH-$CR^{1c}R^{1d}$-CO-, R^{1e}-CO-, or is of the formula:



R^{1b} is hydrogen, alkyl, alkenyl, alkynyl aryl, or a natural or unnatural α-amino acid or a peptide comprising the same;

each of R^{1c} and R^{1d} is independently hydrogen, a radical corresponding to a side chain of a natural and non-natural α-amino acid, alkyl, alkenyl, alkynyl, aryl, or R^{1e}-CO-;

R^{1e} is hydrogen, alkyl, alkenyl, alkynyl, aryl, or R^{1f}-SO_2-;

R^{1f} is alkyl, alkenyl, alkynyl or aryl;

R^N is an amino protecting group;

m is an integer;

each of R^2, R^3, R^4 and R^8 is independently R^{1c} or R^{2a}-CH_2;

R^{2a} is aryl or a heteroalicylic or heteroaromatic group;

R^5 is hydrogen, alkyl, alkenyl, alkynyl or aryl;

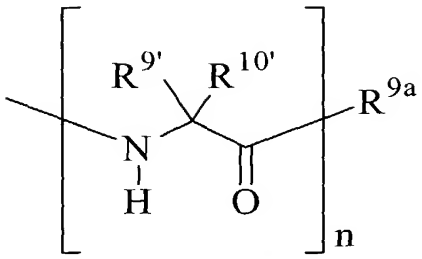
each of R^6 and R^7 is independently hydrogen or alkyl;

R^9 is R^{9a} or R^{9b};

R^{9a} is R^{3a}-X^c-;

R^{3a} is alkyl, alkenyl, alkynyl, aryl, a heteroalicylic or heteroaromatic group, or a group as defined for R^2;

R^{9b} is a group of the formula



each of $R^{9'}$ and $R^{10'}$ is independently as defined above for R^2;

n is an integer;

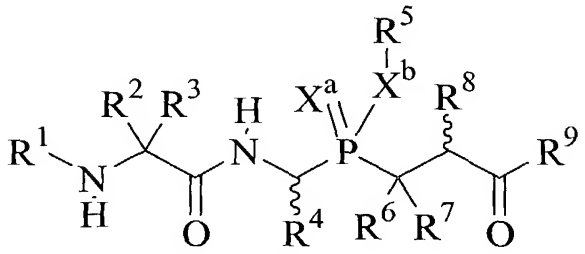
each of X^a, X^b, and X^c is independently O, S, or NH; and

one of R^1, R^2, R^3, R^5 or R^9 optionally incorporates the ligand.

46. (New) An anti-bone resorption agent comprising a compound of claim 33 operatively linked to a ligand targeting the compound of claim 33 to a proteinase involved in bone resorption or to the environment of the proteinase or incorporating such a ligand in or as one of the defined groups R^1, R^2, R^3, R^5 or R^9 of the formula.

47. (New) An anti-bone resorption agent comprising the compound of claim 39 operatively linked to a ligand targeting the compound of claim 39 to a proteinase involved in bone resorption or to the environment of the proteinase.

48. (New) A method of synthesizing a compound of formula (I):



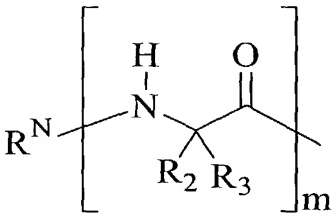
(I)

which comprises contacting a first compound with a second compound under conditions sufficient to provide a compound of formula (I), wherein:

the first compound comprises a phosphorus atom bearing at least the substituents X^a, $-X^b$, $-R^5$, and $-CH(R^4)NH-$;

the second compound comprising a carbon atom bearing the substituents R^6, R^7 and $-C(R^8)-C(O)R^9$;

R^I is hydrogen, an amino-protecting group, alkyl, alkenyl, alkynyl aryl, R^{1b}-NH-$CR^{1c}R^{1d}$-CO-, R^{1e}-CO-, or is of the formula:



R^{1b} is hydrogen, alkyl, alkenyl, alkynyl aryl, or a natural or unnatural α-amino acid or a peptide comprising the same;

each of R^{1c} and R^{1d} is independently hydrogen, a radical corresponding to a side chain of a natural and non-natural α-amino acid, alkyl, alkenyl, alkynyl, aryl, or R^{1e}-CO-;

R^{1e} is hydrogen, alkyl, alkenyl, alkynyl, aryl, or R^{1f}-SO_2-;

R^{1f} is alkyl, alkenyl, alkynyl or aryl;

R^N is an amino protecting group;

m is an integer;

each of R^2, R^3, R^4 and R^8 is independently R^{1c} or R^{2a}-CH_2;

R^{2a} is aryl or a heteroalicylic or heteroaromatic group;

R^5 is hydrogen, alkyl, alkenyl, alkynyl or aryl;

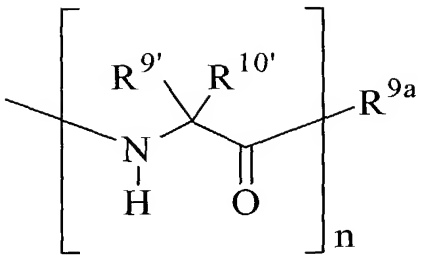
each of R^6 and R^7 is independently hydrogen or alkyl;

R^9 is R^{9a} or R^{9b};

R^{9a} is R^{3a}-X^c-;

R^{3a} is alkyl, alkenyl, alkynyl, aryl, a heteroalicylic or heteroaromatic group, or a group as defined for R^2;

R^{9b} is a group of the formula



each of $R^{9'}$ and $R^{10'}$ is independently as defined above for R^2;

n is an integer; and

each of X^a, X^b, and X^c is independently O, S, or NH.

49. (New) The method of claim 48, wherein the second compound is attached to a solid support.

50. (New) The method of claim 49, wherein the second compound is attached to the solid support by an acylation of a primary amino group attached to the solid support.

51. (New) The method of claim 50, further comprising solid phase peptide synthesis on the solid support to provide the primary amino group.

52. (New) The method of claim 48, further comprising solid phase peptide synthesis from the amino group in the -CH(R^4)NH- substitutent of the second compound. --

REMARKS

Claims 1-27 have been canceled without prejudice to Applicants' right to pursue them in one or more continuations, divisionals, or continuations-in-part of this application. New claims 28-52, all of which are supported by the application as originally filed, are now pending in this application. No new matter has been added.

No fee is believed due for this preliminary amendment. If any fee is due, please charge such fee to Pennie & Edmonds LLP Deposit Account No. 16-1150.

Respectfully submitted,

Date March 21, 2002

45,479

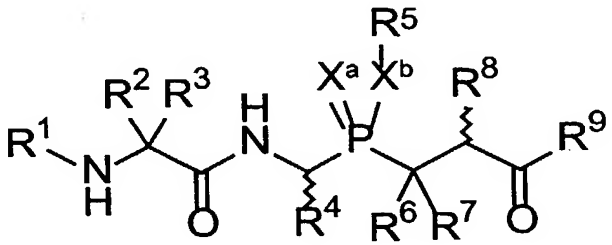
Max Bachrach (Reg. No.)

For: Paul J. Zegger (Reg. No. 33,821)

PENNIE & EDMONDS LLP
1667 K Street, N.W.
Washington, DC 20006
(202) 496-4400

SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES

The present invention relates to substituted phosphinate based peptide derivatives of formula (I) which are useful in the treatment of metalloproteinase-mediated conditions such as metabolic bone diseases, including but not limited to osteoporosis and bone metastasis.



(I)

The invention also relates to a method of using these compounds in a pharmaceutical composition suitable therefore in the treatment of the above diseases in mammals, especially humans. In addition, the compounds may be used in combination therapy with e.g., radiotherapy, gene therapy, or drugs for hormone replacement therapy, bone anabolic agents, cytotoxic drugs, and analgesics. Novel compounds within formula I are described which have both similar and broader utility.

Human bone is constantly undergoing remodelling. The fine balance between bone resorption and bone formation is regulated by local and systemic factors and by physical forces acting on various cells including, in the bone environment, the osteoclast and the osteoblast as well as specialised forms of the latter such as the bone lining cell and the osteocyte. However, in

several metabolic bone diseases including most importantly osteoporosis and osteolytic bone metastasis, the balance is disturbed resulting in a sustained pathological net bone resorption.

Osteoporosis is a systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a subsequent increase in bone fragility and susceptibility to fracture. Post-menopausal osteoporosis is a chronic disease which affects millions of women throughout the world and it has an enormous economical and social impact on society.

Bone metastasis results from spreading of primary tumours to bone, where the cancer cells can interfere with the normal bone remodelling process through local regulation of osteoblast and/or osteoclast activities. This may subsequently lead to acute focal excess of bone formation over bone resorption or vice versa as in the case of osteosclerotic and osteolytic metastasis, respectively. Breast cancer is the most frequent non-skin cancer in the female population of industrialised countries with approx. 500,000 new cases per year. Between 25% and 75% of these patients develop osteolytic bone metastases, and 90% of those patients who die of breast cancer have bone metastases. This severe prognosis makes treatment mandatory, and in most cases preventive measures are taken already at the time of identification of the primary tumour. Other primary tumours that frequently spread to cause osteolytic bone metastasis include those associated with prostate cancer and myelomatosis.

Reduction of bone resorption is believed to be an appropriate way to prevent and treat several metabolic bone diseases, including osteoporosis and osteolytic bone metastasis. Agents such as calcitonin and bisphosphonates are able to suppress bone resorption and have been used for prevention and

treatment of osteoporosis and/or osteolytic bone metastasis. Furthermore, the use of steroid hormones (especially oestrogen) in hormone replacement therapy is an established prophylactic method for post-menopausal osteoporosis. However, these therapeutic agents fail to achieve satisfactory effects in some cases, due to subject limitation or uncertain efficacy, and particularly for preventive medication in osteoporosis risk groups compliance is low. Furthermore, there is not yet a curative treatment for bone metastasis, and all currently used measures for bone metastatic patients are of palliative type. There is therefore need for a new prophylactic/therapeutic method for preventing or treating accentuated bone resorption.

Removal of the mineralised osseous substance, i.e. organic matrix embedded in deposits of calcium phosphate salts, is a complicated process. Though still a controversial subject, it appears that osteoclasts are the only cells capable of bone resorption. The progressing bone loss in patients with osteoporosis as well as the acute local bone loss at metastatic sites in bones of patients with osteolytic metastasis are caused by increased osteoclast activity.

The expected life cycle of osteoclasts involves the following major phases:

1. recruitment of early osteoclast precursors from haematopoietic stem cells,
2. proliferation of osteoclast precursors,
3. differentiation of osteoclast precursors and maturation including fusion of mononuclear into multinuclear cells,
4. migration to bone, often through a layer of bone lining osteoblasts to reach the resorptive bone surface,
5. attachment to and polarisation on the resorptive bone surface,

6. removal of mineralised osseous substance through secretion of protons and proteolytic enzymes into the sub-osteoclastic resorption zone, and
7. death by apoptosis, necrosis or a more random process.

These phases are, however, not always temporally (or spatially) separate events, e.g. differentiation might take place during migration to the resorptive surface and fusion might take place on the bone surface. All these phases represent possibilities for intervention in order to regulate the level of bone resorption.

Traditionally, proteolytic enzymes have been known to play a role in the degradation of the organic matrix of bone, and in particular in the removal of its type I collagen fibres. Therefore, the speculations about the biological roles of proteinases in bone have almost entirely focused on proteinases of osteoclast origin and their potential ability as to degrade organic bone matrix in the sub-osteoclastic resorption zone. However, we show here that, though proteinases of osteoclast origin are of central importance, proteinases produced by neighbouring cells such as osteoblasts and bone-metastasising cancer cells also have important influences on bone metabolism. Furthermore, our recent findings have shown that proteolytic enzymes are not only acting in the resorption zone but are also very important for the migration and attachment of osteoclasts to the resorptive surface (Blavier & Delaissé, 1995, Sato *et al*, 1998). In addition, proteinase-dependent migration of immature osteoclasts appears to be associated with the maturation and fusion into active bone-resorbing osteoclasts, i.e. osteoclast differentiation processes.

Interference with an early phase of the osteoclast life cycle e.g., osteoclast migration and/or attachment in the

recruitment process by a proteinase inhibitor might be more effective in impairing bone resorption than inhibition of a proteinase involved directly in the resorptive process. The former type of interference will also be easier to accomplish since the secreted enzymes of the migrating osteoclasts or osteoclast-precursors are not protected from inhibition as they are when secreted into the tightly sealed resorption zone which is formed when the active polarised osteoclasts attach to bone. Furthermore, according to our novel findings, proteolytic enzymes of osteoblasts, including bone lining cells and osteocytes, are involved in the regulation of the passage of osteoclasts to the resorptive bone surface and in the removal of proteins, in particular collagen fibres, which remain in the resorption lacunae after the osteoclastic resorption process has ended. Thus, inhibition of proteinases produced by osteoblasts including bone lining cells and osteocytes may be an effective method for regulation of bone metabolism in general, and for reducing osteoclastic bone resorption in particular.

The knowledge about proteolytic enzymes involved in bone resorption mainly comes from studies of the effects of natural and particularly synthetic enzyme inhibitors in bone cell and tissue cultures. Furthermore, histochemical and immunocytochemical characterisation of enzymes expressed by bone cells and tissues *in vitro* or *in vivo*, as well as more recently identification of enzyme-encoding mRNA in osteoclasts and other bone cells has increased the information about the particular proteolytic enzymes involved in bone resorption. Recently, the development of several strains of transgenic mice deficient for different particular proteinases has improved the possibility to clarify their roles in bone both *in vivo* and after cell and tissue isolation and culture.

The proteolytic enzymes of major relevance to osteoclastic bone resorption are members of the families of cysteine proteinases and metalloproteinases, of which some of the most important appear to be cathepsin K and L (EP-A-0611756) as well as several of the Zn-containing matrix metalloproteinases (MMPs), respectively. Of the currently known approx. 15 structurally related, but genetically different members of the human MMP-family, at least MMP-9, and the membrane-type 1 MMP (MT1-MMP or MMP-14 as it is also called) and possibly also MMP-1 and MMP-12 are expressed by osteoclasts. Furthermore, the most important MMPs in osteoblasts appear to be MMP-2, MMP-13 and possibly MMP-1, MMP-11 and MMP-14. The different members of the MMP-family play distinct roles in degradation of extracellular molecules under both physiological and pathological conditions. They are all produced as latent proenzymes and most MMPs, except MMP-11, MMP-23 and the transmembrane MMPs (including apart from MMP-14, MT2-MMP or MMP-15, MT3-MMP or MMP-16, and MT4-MMP or MMP-17), are activated extracellularly by an auto-catalytic mechanism and/or in complex cascade reactions by other proteinases including MMPs. In addition to their structural relatedness, there is a large homology between the primary sequences of the various MMPs. This is in particular the case for the well-conserved catalytic domains of the various members of the MMP-family; a fact that renders the development of inhibitors selective for one or a subset of MMPs difficult.

Most of the synthetic proteinase inhibitors developed over the last 10 years have been based on a substrate-mimicking peptide or pseudo-peptide framework. In the case of MMPs, the majority of novel inhibitors have included a Zn-binding group, such as a hydroxamate or carboxylate. These MMP inhibitors have, however, mainly been able to interact with either the unprimed (P-), or more frequently the primed (P'-) side of the catalytic

cleft of the proteinase, but not with both sides. This limitation does not seem to reduce the opportunity to produce high affinity MMP inhibitors with K_i-values in the low or even sub-nanomolar range, but does reduce the possibilities of creating a selective inhibition of just one particular or a subset of the MMPs. Furthermore, the most widely studied and very potent hydroxamate-type MMP inhibitors such as batimastat (BB-94) and galardin (GM6001) display poor solubility, unfavourable pharmacokinetics and/or toxic side effects.

Phosphinate-based peptide derivatives of the formula (I) represent an alternative way to prepare molecules, which mimic the peptidic conformation of metalloproteinase substrates and chelate its catalytic Zn. In these derivatives, a peptide bond (-CO-NH-) susceptible to enzymatic hydrolysis has been replaced by a phosphinate group ($-P(O)(OH)-CH_2-$). There are several advantages associated with these structures compared to other types of metalloproteinase inhibitors such as peptido-mimicking hydroxamates or carboxylates. The transition state of a peptide bond undergoing hydrolysis by a metalloproteinase is typically depicted as $-C(O^-)(OH)-N^+H_2-$ or $-C(OH)_2-NH-$ indicating its structural resemblance to the phosphinate group. A further indication, that phosphonamidate-type as well as phosphinate-type metalloproteinase inhibitors act as transition state analogues, rather than multisubstrate ground state analogues, was given by Bartlett and Marlow (1983), who showed that the k_{cat}/K_m values, but not the K_m values, of peptidic thermolysine substrates, were highly correlated to the K_i values of the corresponding phosphonamidate-type inhibitors. Furthermore, the use of a phosphinate bond, in contrast to hydroxamates and carboxylates, allows substrate mimicking at both the P- and P'-corresponding sides of the inhibitor, and thereby an improved opportunity for increasing its selectivity towards particular

proteinases. Finally, the dipeptido-mimetic nature of the P1-P1' corresponding part of the molecule, i.e. according to the nomenclature of formula (I), the dipeptido-mimetic, $-NH-CH(R^4)-P(X^a)(X^b-R^5)-C(R^6)(R^7)-CH(R^8)-CO-$, does not only allow the use of traditional methods for peptide synthesis in the construction of individual inhibitors but also the use of combinatorial inhibitor libraries by including the dipeptido-mimetic in a protected building block format in the same way as amino acids are used.

Alternatively, the phosphinic dipeptido-mimetic may be incorporated into peptides by solid phase synthesis using a direct approach, in which two building blocks are used, one containing X^a, X^b, R^4 and R^5, and one containing R^6, R^7 and R^8. This methodology provides the possibility for the generation of compounds of formula (I) directly on the solid phase.

Phosphinic acid derivatives of a traditional peptide framework (EP-0276436, US 5,776,903) have been described earlier, but not as regulators of metabolic bone diseases. More sophisticated substituted phosphinate based peptide derivatives having a substituted aryl in the P1'-corresponding side chain (US 5,579,700) or at the P-corresponding side of the inhibitor rather than a peptide or peptide-like sequence (WO98/03516) have been claimed for general use in the treatment of diseases mediated by MMP-3 and diseases characterised by MMP-activity, respectively.

The design of synthetic inhibitors has traditionally been based on the cleavage site of peptide substrates. The optimisation process aiming at increased inhibitor potency (characterised by reduction of the K_i-values) and/or increased inhibitor selectivity (characterised by K_i-ratios several orders of magnitude from 1) has mainly been done by simple side chain substitutions typically in the form of amino acid permutations

in peptide derivatives (i.e., single mutation experiments). However, the subsites of proteinase inhibitors are not independent, e.g. two mutations which individually add beneficially to the characteristics of an inhibitor very often lead to less than additive effects if combined. Thus, it is very difficult to develop potent and selective proteinase inhibitors through repeated and combined single mutation syntheses.

The recent progress in development of synthetic compound libraries containing a large number of different compounds prepared by e.g. combinatorial chemistry has provided efficient alternatives for the development of proteinase substrates and inhibitors. We have developed permeable resins consisting of polyethylene glycol-polyacrylamide copolymers (so-called PEGA resins, see WO93/16118), which when prepared in the form of small spherical beads with a typical diameter of approx. 0.1 mm, can be used for solid-phase combinatorial (pseudo-)peptide synthesis of large numbers of potential proteinase substrates and inhibitors (Meldal *et al*, 1994; and Renil *et al*, 1998). These PEGA bead libraries allow the identification of the most potent among several hundred thousand compounds in a single step incubation with a few ml of approx. 10-100 nM proteinase.

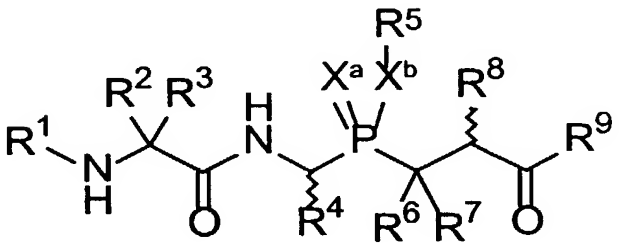
The identification of a suitable synthetic proteinase inhibitor such as a phosphinate based peptide derivative may be followed by appropriate modification of this compound to assure its use as a medicament for the treatment of metabolic bone disease. Several characteristics are necessary, particularly sufficient uptake and stability in the living organism to assure a beneficial effect, sufficient tissue or cell specific action to assure maximal effects at the target site of the organism relative to effects at non-target sites including acceptable levels of side effects, and a pharmacologically acceptable dose- and time-response to the treatment.

Administration of proteins, peptides and peptide-like substances to animals and humans requires protective routes of administration and/or protective formulation of the peptide in order to avoid degradation of the compound. Though protective encapsulation for oral administration of peptides and peptide-like agents is a technology currently undergoing significant improvement, stabilisation of the agent itself prior to administration is advantageous. For peptide-mimicking MMP-inhibitors this has been possible by chemical modification of an initially identified compound apparently without important changes in its inhibitory capacity (Brown & Giovazzi, 1995 and P. D. Brown personal communications June 1996).

Targeting of a proteinase inhibitor to a particular cell type, e.g. osteoclasts, or particular tissue, e.g. bone, can be obtained by two general means. One, is if the inhibitor due to its intrinsic specificity selectively reacts with the proteinase associated with this cell type or tissue either because the proteinase at this target cell type or tissue is particularly available to the inhibitor (due to e.g. the localisation of the cell or tissue, the localisation of the proteinase in the cell or tissue, or simply by a local high concentration of the proteinase) or because the proteinase when associated with this cell type or tissue is different from the corresponding proteinase as it is expressed when associated with other cell types and tissues (due to e.g. immobilisation or post-translational modifications). The other way to obtain a specificity is by making hybrid molecules or conjugates combining one part of the agent having proteinase-inhibitory characteristics with another part having antibody or ligand specificity for the particular cell type or tissue. These hybrids can be made by recombinant expression of fusion-proteins after cloning of a hybrid cDNA. E.g., a piece of cDNA encoding the osteoclast-specific ligand calcitonin (or a receptor-binding part thereof) can be ligated to another piece of cDNA encoding a peptide inhibitor for an osteoclast proteinase. Hybrids can also be conjugates of two compounds, e.g. by chemically linking an

amino-bisphosphonate, which has high affinity for hydroxyapatite in bone, or an antibody specific for a component exposed in the osteoclast membrane, such as the calcitonin receptor with a peptide or peptide-mimicking proteinase inhibitor.

The present invention includes compounds of the formula (I):



(I)

or a pharmaceutically acceptable salt thereof, where:

R^1 is

1. a hydrogen atom
2. an amino protecting group such as a group R^{1a}-O-CO- in which R^{1a} is
   2.1. an optionally substituted alkyl group (preferably C_1 to C_{10} more preferably C_1 to C_6 and optionally substituted with e.g. hydroxy halogen (e.g. chlorine or fluorine), trifluoromethyl, or hydroxy alkyl) such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, neopentyl, tert-pentyl, 1-ethylpropyl, norbonyl, hexyl, isohexyl, 1,1-dimethylbutyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1-adamantyl, 2-adamantyl, 2-ethylbutyl, heptyl, octyl, nonyl, decyl or an alkyl group, preferably having from 1

to 10 (more preferably 1 to 6) carbon atoms in the alkyl moiety), optionally substituted in the aryl moiety by alkyl (preferably C_1 to C_6), hydroxy, alkoxy (preferably C_1 to C_6) such as benzul, 2,4,6-trimethylbenzyl, 4-tert-butylbenzyl, 4-tert-butoxybenzyl, 4-hydroxybenzyl, 4-methoxybenzyl, 2,4-dimethoxybenzyl, 3,4-dihydroxybenzyl, 3,4-dimethoxybenzyl, 9-fluorenylmethyl, etc.

2.2. an alkenyl group (preferably C_1 to C_{10}, more preferably C_1 to C_6) such as vinyl, allyl, isopropenyl, 1-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 2-ethyl-1-butenyl, 3-methyl-2-butenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 4-methyl-3-pentenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl and 5-hexenyl, 2-cyclopenten-1-yl, 3-cyclopenten-1-yl, 2-cyclohexen-1-yl and 3-cyclohexen-1-yl, etc.

2.3. an alkynyl group such (preferably C_1 to C_{10}, more preferably C_1 to C_6) such as ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 4-pentynyl, 1-hexynyl, 2-hexynyl, 3-hexynyl, 4-hexynyl and 5-hexynyl, etc.

2.4. an aryl group such as phenyl, naphthyl, anthryl, phenanthryl, acenaphthylenyl, fluorenyl.

2.5. an aryl group such as phenyl, naphthyl, anthryl, phenanthryl, acenaphthylenyl, fluorenyl which is fluorinated or chlorinated in one or more positions.

3. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

4. a group R^{1b}-NH-$CR^{1c}R^{1d}$-CO- in which

4.1. R^{1b} is

4.1.1. a hydrogen atom.

4.1.2. a natural or non-natural α-amino acid such as alanine, arginine, asparagine, aspartic acid,

cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, norleucine, lysine, methionine, phenylalanine, proline, hydroxyproline, hydroxylysine, serine, threonine, tryptophan, tyrosine, valine or a peptide consisting of the same.

4.1.3. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

4.2. R^{1c} and R^{1d} independently of each other are

4.2.1. a hydrogen atom.

4.2.2. a radical corresponding to natural and non-natural α-amino acids such as alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, norleucine, lysine, methionine, phenylalanine, proline, hydroxyproline, hydroxylysine, serine, threonine, tryptophan, tyrosine, valine, nitrophenyl alanine, 3-nitrotyrosine, homoarginine, thiazolidine, dehydroproline, homocysteine, α-aminobutyric acid, α-aminoisobutyric acid, 2-aminobenzoic acid, 4-aminobenzoic acid, homoalanine, norvaline, ornithine, phenylglycine, pyroglutamic acid, sarcosine, etc.

4.2.3. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

5. a group R^{1e}-CO- in which R^{1e} is

5.1. a hydrogen atom.

5.2. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

6. a group R^{1f}-SO_2- in which R^{1f} is an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

R^2, **R^3**, **R^4** and **R^8** independently of each other are

1. a group as R^{1c}.

2. a group R^{2a}-CH_2- in which R^{2a} is

2.1. an aryl group such as phenyl, naphthyl, anthryl, phenanthryl, acenaphthylenyl, fluorenyl

2.2. a heteroalicylic or heteroaromatic group such as pyrrolidyl, piperidyl, morpholino, furyl, thienyl, pyrrolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, imidazolyl, pyrazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,3,4-oxadiazolyl, furazanyl. 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,3,4-thiadiazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, tetrazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl and triazinyl, benzofuranyl, isobenzofuranyl, benzo(b)thienyl, indolyl, isoindolyl, 1H-indazolyl, benzimidazolyl, benzoxazolyl, 1,2-benzisoxazolyl, benzothiazolyl, 1,2-benzisothiazolyl, 1H-benzotriazolyl, quinolyl, isoquinolyl, cinnolinyl, quinazolinyl, quinoxalinyl, phthalazinyl, naphthylizinyl, purinyl, pteridinyl, carbazolyl, a-carbolinyl, B-carbolinyl, g-carbolinyl, acricinyl, phenoxazinyl, phenothiazinyl, phenazinyl, phenoxthinyl, thianthrenyl, phenanthridinyl, phenanthrolinyl, indolizinyl, etc.

R^5 is

1. a hydrogen atom.
2. a group as R^{1a}.

R^6 and **R^7** independently of each other are

1. a hydrogen atom.
2. an alkyl group such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, etc.

R^9 is

1. a group R^{3a}-X^c- in which
   1.1. R^{3a} is

1.1.1. a group as R^{1a}.

1.1.2. a heteroalicylic or heteroaromatic group such as pyrrolidyl, piperidyl, morpholino, furyl, thienyl, pyrrolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, imidazolyl, pyrazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,3,4-oxadiazolyl, furazanyl. 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,3,4-thiadiazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, tetrazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl and triazinyl, benzofuranyl, isobenzofuranyl, benzo(b)thienyl, indolyl, isoindolyl, 1H-indazolyl, benzimidazolyl, benzoxazolyl, 1,2-benzisoxazolyl, benzothiazolyl, 1,2-benzisothiazolyl, 1H-benzotriazolyl, quinolyl, isoquinolyl, cinnolinyl, quinazolinyl, quinoxalinyl, phthalazinyl, naphthylizinyl, purinyl, pteridinyl, carbazolyl, a-carbolinyl, B-carbolinyl, g-carbolinyl, acricinyl, phenoxazinyl, phenothiazinyl, phenazinyl, phenoxthinyl, thianthrenyl, phenanthridinyl, phenanthrolinyl, indolizinyl, etc.

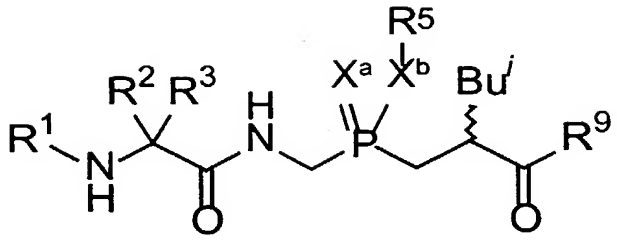
1.1.3. a group as $\mathbf{R^2}$.

1.2. X^c is O, S or NH.

$\mathbf{X^a}$ and $\mathbf{X^b}$ is:

1. O
2. S
3. NH

One genus of this embodiment is the compounds of the formula (II):



(II)

or a pharmaceutically acceptable salt, ester or amide thereof, where R^1, R^2, R^3, R^5, R^9, X^a amd X^b are as defined in Formula I, i.e.

R^1 is

1. a hydrogen atom
2. a group R^{1a}-O-CO- in which R^{1a} is
   - 2.1. an alkyl such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, neopentyl, tert-pentyl, 1-ethylpropyl, norbonyl, hexyl, isohexyl, 1,1-dimethylbutyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1-adamantyl, 2-adamantyl, 2-ethylbutyl, heptyl, octyl, nonyl, decyl, benzyl, 2,4,6-trimethylbenzyl, 4-tert-butylbenzyl, 4-tert-butoxybenzyl, 4-hydroxybenzyl, 4-methoxybenzyl, 2,4-dimethoxybenzyl, 3,4-dihydroxybenzyl, 3,4-dimethoxybenzyl, 9-fluorenylmethyl, etc.
   - 2.2. an alkenyl group such as vinyl, allyl, isopropenyl, 1-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 2-ethyl-1-butenyl, 3-methyl-2-butenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 4-methyl-3-pentenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl and 5-hexenyl, 2-cyclopenten-1-yl, 3-cyclopenten-1-yl, 2-cyclohexen-1-yl and 3-cyclohexen-1-yl, etc.

2.3. an alkynyl group such as ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 4-pentynyl, 1-hexynyl, 2-hexynyl, 3-hexynyl, 4-hexynyl and 5-hexynyl, etc.

2.4. an aryl group such as phenyl, naphthyl, anthryl, phenanthryl, acenaphthylenyl, fluorenyl.

2.5. an aryl group such as phenyl, naphthyl, anthryl, phenanthryl, acenaphthylenyl, fluorenyl which is fluorinated or chlorinated in one or more positions.

3. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

4. a group R^{1b}-NH-$CR^{1c}R^{1d}$-CO- in which

4.1. R^{1b} is

4.1.1. a hydrogen atom.

4.1.2. a natural α-amino acid such as alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, norleucine, lysine, methionine, phenylalanine, proline, hydroxyproline, hydroxylysine, serine, threonine, tryptophan, tyrosine, valine or a peptide consisting of the same.

4.1.3. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

4.2. R^{1c} and R^{1d} independently of each other are

4.2.1. a hydrogen atom.

4.2.2. a radical corresponding to natural and non-natural α-amino acids such as alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, norleucine, lysine, methionine, phenylalanine, proline, hydroxyproline, hydroxylysine, serine, threonine, tryptophan, tyrosine, valine, nitrophenyl alanine, 3-nitrotyrosine, homoarginine, thiazolidine, dehydroproline, homocysteine, α-

aminobutyric acid, α-aminoisobutyric acid, 2-aminobenzoic acid, 4-aminobenzoic acid, homoalanine, norvaline, ornithine, phenylglycine, pyroglutamic acid, sarcosine, etc.

4.2.3. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

5. a group R^{1e}-CO- in which R^{1e} is

5.1. a hydrogen atom.

5.2. an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

6. a group R^{1f}-SO_2- in which R^{1f} is an alkyl, alkenyl, alkynyl or aryl group as R^{1a}.

R^2 and **R^3** independently of each other are

1. a group as R^{1c}.

2. a group R^{2a}-CH_2- in which R^{2a} is

2.1. an aryl group such as phenyl, naphthyl, anthryl, phenanthryl, acenaphthylenyl, fluorenyl

2.2. a heteroalicylic or heteroaromatic group such as pyrrolidyl, piperidyl, morpholino, furyl, thienyl, pyrrolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, imidazolyl, pyrazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,3,4-oxadiazolyl, furazanyl. 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,3,4-thiadiazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, tetrazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl and triazinyl, benzofuranyl, isobenzofuranyl, benzo(b)thienyl, indolyl, isoindolyl, 1H-indazolyl, benzimidazolyl, benzoxazolyl, 1,2-benzisoxazolyl, benzothiazolyl, 1,2-benzisothiazolyl, 1H-benzotriazolyl, quinolyl, isoquinolyl, cinnolinyl, quinazolinyl, quinoxalinyl, phthalazinyl, naphthylizinyl, purinyl, pteridinyl, carbazolyl, a-carbolinyl, B-carbolinyl, g-carbolinyl, acricinyl, phenoxazinyl,

phenothiazinyl, phenazinyl, phenoxthinyl, thianthrenyl, phenanthridinyl, phenanthrolinyl, indolizinyl, etc.

R^5 is

1. a hydrogen atom.
2. a group as R^{1a}.

R^9 is

1. a group R^{3a}-X^c- in which
   1.1. R^{3a} is
      1.1.1. a group as R^{1a}.
      1.1.2. a heteroalicylic or heteroaromatic group such as pyrrolidyl, piperidyl, morpholino, furyl, thienyl, pyrrolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, imidazolyl, pyrazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,3,4-oxadiazolyl, furazanyl. 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,3,4-thiadiazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, tetrazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl and triazinyl, benzofuranyl, isobenzofuranyl, benzo(b)thienyl, indolyl, isoindolyl, 1H-indazolyl, benzimidazolyl, benzoxazolyl, 1,2-benzisoxazolyl, benzothiazolyl, 1,2-benzisothiazolyl, 1H-benzotriazolyl, quinolyl, isoquinolyl, cinnolinyl, quinazolinyl, quinoxalinyl, phthalazinyl, naphthylizinyl, purinyl, pteridinyl, carbazolyl, a-carbolinyl, B-carbolinyl, g-carbolinyl, acricinyl, phenoxazinyl, phenothiazinyl, phenazinyl, phenoxthinyl, thianthrenyl, phenanthridinyl, phenanthrolinyl, indolizinyl, etc.
      1.1.3. a group as R^2.
   1.2. X^c is O, S or NH.

X^a and X^b is:

1. O
2. S
3. NH

Compounds of Formula II are novel per se and will find utility in regulation of bone metabolism, regulation of metalloproteinases, regulation of MMPs, regulators of other proteinases, and of proteinase mediated diseases.

Exemplifying the invention are the 91 compounds (named 01-01 to 01-07, 02-01 to 02-22, 03-01, 04-01 to 04-21, and 05-01 to 05-40) each in two stereoisomeric forms (named A and B) listed in Table 1 of Example 5, Table 2 of Example 6, Table 3 of Example 8, and Table 4 of Example 9.

This invention also relates to a pharmaceutical composition for treatment of metalloproteinase-mediated metabolic bone diseases, including but not limited to osteoporosis and bone metastasis incorporating an amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof effective in such treatments and a pharmaceutically acceptable carrier.

The present invention also provides the use of an agent in the manufacture of a medicament for the treatment of metalloproteinase-mediated metabolic bone diseases, including but not limited to osteoporosis and bone metastasis in a mammal, including a human, comprising administering to said mammal an amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof effective in such treatments and a pharmaceutically acceptable carrier.

The invention will be further described and illustrated but not limited by the details thereof in the examples which follow and the appended drawings in which:

Figure 1 shows the reaction scheme for preparation of the building block **III** used to prepare compounds of the formula (I) containing a phosphinic dipeptido-mimetic moiety by solid phase synthesis. Reactions are Step A: Michael addition of an α-amino phosphinic acid to an acrylic ester to form the phosphorus-carbon bond, Step B: esterification of the phosphinic acid, Step C: protective group manipulations. R^N may be any amino protective group, preferably Fmoc, Alloc, Cbz or Boc. R^4, R^5, R^6, R^7, and R^8 may be any group according to the descriptions of the corresponding groups of compounds of the formula (I). R^C may be any carboxy protective group, preferably an ethyl or any other group included in R^{1a} of compounds of the formula (I).

Figure 2 shows the general reaction scheme for preparation by solid phase synthesis of those compounds of the formula (I) in which R^1 and R^9 represent amino acids or peptides.. Reactions are: Step A: attachment of the 4-hydroxymethylbenzoyl linker, Step B: coupling of amino acids by conventional peptide synthesis, Step C: coupling of the building block **III**, Step D: coupling of amino acids by conventional peptide synthesis, Step E: cleavage of the compound off the solid support. R^1, R^2, R^3, R^4, R^5, R^6, R^7, and R^8 may be any group according to the descriptions of the corresponding groups of compounds of the formula (I). $R^{9'}$ and $R^{10'}$ may be any group as R^2; R^{11} may be any group as R^{1a}; R^N may be any amino protective group, preferably Fmoc, Alloc, Cbz or Boc.

Figure 3 shows the general reaction scheme for the preparation of compounds of formula (I) by the direct approach on solid phase. Reactions are: Step A: solid phase peptide synthesis, Step B: acryloylation of the N-terminus, Step C:

addition of a phosphinic acid to the acrylamide, Step D: removal of R^N and subsequent solid phase peptide synthesis, Step E: cleavage of the compound from the solid support. R^1, R^2, R^3, R^4, R^5, R^6, R^7, and R^8 may be any group according to the descriptions of the corresponding groups of compounds of the formula (I). $R^{9'}$ and $R^{10'}$ may be any group as R^2; R^{11} may be any group as R^{1a}; R^N may be any amino protective group, preferably Fmoc, Alloc, Cbz or Boc.

Figure 4 shows the reaction scheme for preparation of a one-bead-two-compounds solid phase combinatorial library of phosphinate based peptidic compounds of the formula (II) containing the phosphinic Gly-Leu dipeptido-mimetic moiety for screening against matrix metalloproteinases. One-letter abbreviations for amino acids are used. Reactions are Step A: introduction of a functional biantenna consisting a free amino group and an Alloc protected amino group, Step B: attachment of a photolabile linker (Holmes and Jones, *1995)* and a mass spacer hexapeptide sequence which is common for all beads, Step C: library synthesis (X^1 to X^6 represent amino acids) by the split and combine methodology using 10% ladder capping, Step D: coupling of the building block **III**, Step C repeated, Step E removal of the Alloc group and coupling of a protected general MMP-substrate.

Figure 5 shows the fragments formed by ladder capping in the synthesis of the library. X^1 to X^6 represent amino acids. From the differences between the masses of these fragments the sequence of an active inhibitor was determined.

Figure 6 shows the frequencies of the different amino acids in the different subsites of the inhibitors found on the 82 dark PEGA beads after incubation with MMP-12.

Figure 7 schematically shows the sequential incubations of the one-bead-two-compounds solid phase combinatorial library of

phosphinate based peptidic compounds of the formula (II). The trapezoids represent batch incubations with either 370 nM MMP-9, 100 nM MMP-13 or 100 nM MMP-14 for either 24 hrs (primary proteinase incubation), or with 200 nM MMP-9, 100 nM MMP-13 or 100 nM MMP-14 for 1 hr (secondary and tertiary incubations). The arrows indicate transfer of dark beads. The numbers in brackets at the base of each trapezoid representing a secondary and tertiary incubation, e.g. (+9+13-14), designate the name of the group of selected beads, and indicate the selectivity of the phosphinate based peptidic compounds belonging to that group. E.g., compounds on beads belonging to group (+9+13-14) seem to be capable of inhibiting both MMP-9 and MMP-13, but not MMP-14. The order of the numbers in the bracket describes the order by which the sequential incubations were made. The numbers in bold below indicate the total number of fluorescent beads that were isolated for each group. E.g., group (+9+13-14) included 12 beads that remained dark after incubation with first MMP-9 and then MMP-13, but turned fluorescent when subsequently incubated with MMP-14. All 209 selected beads belonging to the 12 groups were used for MALDI-TOF sequence analysis of the corresponding phosphinate based peptide derivatives. However, the 78 sequences belonging to the two groups (+14-9) and (+14-13) were possibly false positives and not used for further analysis. Among the remaining 131 sequences, 4 sequences for each of the other 10 groups were selected for synthesis.

Figure 8 shows the effect of different proteinase inhibitors on osteoclast invasion through a type I collagen matrix *in vitro*. All synthetic MMP inhibitors (at 10 μM), including the hydroxamate-type inhibitors, RP59794, BB-94 (K_i = 0.3 nM for MMP-9), and GM6001 (K_i = 1 nM), and the novel phosphinate-type inhbitors, 01-02B (K_i = 70,000 nM for MMP-9), 01-01A (K_i = 300 nM), 01-07B (K_i = 80 nM), and 01-07A (K_i = 1.2

nM), as well as the natural TIMP-2, reduced the invasion of osteoclasts. In contrast neither the cysteine proteinase inhibitors, E64 and EST, nor the serine proteinase inhibitor aprotinin (at 10-40 μM) showed any inhibitory effect, indicating the specific role of MMPs in the invasive process.

Figure 9 shows the pericellular collagenolytic activity exerted by osteoclasts seeded on a type I collagen surface in the presence or absence of 10 μM of the hydroxamate-type MMP inhibitor, GM6001.

Figure 10 shows the significant effect of 50 μM of the hydroxamate-type MMP inhibitor, GM6001, on the migrated distance of osteoclasts seeded on a type I collagen surface and observed for 4 to 12 hours. *: $p<0.05$, **: $p<0.01$.

Figure 11 shows the inhibitory effect on bone resorption exerted by a bone lining cell layer. A confluent cell layer of osteoblastic lining cells shielded the bone and thereby reduced the access of osteoclasts to the bone surface. The resorption was further reduced by addition of the hydroxamate-type MMP inhibitor, GM6001.

Figure 12 shows the significant inhibition ($p<0.001$) of the TGF-β induced elongation of osteoblasts in a confluent cell layer by the hydroxamate-type MMP inhibitor, GM6001 (10 μM). In contrast the inhibitors of serine proteinases, cysteine proteinases, and aspartic proteinases, i.e. aprotinin, E-64, and pepstatin (all 10 μM), respectively, did not affect the osteoblast elongation when compared to the TGF-β treated control without proteinase inhibitor. All values are Mean+SD (n=9) of the relative cell free surface area of the culture dish in each of 3 experiments.

Figure 13 shows the significant reduction ($p<0.0001$) in the TGF-β induced elongation of osteoblast in a confluent cell

layer by the hydroxamate-type MMP inhibitor, GM6001 (10 μM), and by the novel phosphinate-type MMP inhibitor, 01-07A (10 μM), when compared to the TGF-β treated control without proteinase inhibitor. All values are Mean+SD (n=9) of the relative cell free surface area of the culture dish.

Figure 14 shows the inhibition by the MMP inhibitor GM6001 of the TGF-β induced increase in bone resorption when the bone lining cell layer on bone slices was treated simultaneously with 2.5 ng/ml TGF-β and 10 μM GM6001 before fixation and seeding of osteoclasts. All values are Mean+SD (n=4) in each of 2 experiments.

Figure 15 shows that decalcification was significantly stimulated by 2.5 ng/ml TGF-β in cultures of calvariae from 18 day-old mouse foetuses. All values are Mean+SD (n=5).

Figure 16 shows that the novel phosphinate-type MMP inhibitor, 01-07A (10 μM), when added simultaneously with 2.5 ng/ml TGF-β to cultures of calvariae from 18 day-old mouse foetuses markedly reduce their decalcification. All values are Mean+SD (n=5).

Figure 17 shows the inhibitory effect of the hydroxamate-type MMP inhibitor, BB-94, on the decalcification of cultured metatarsals and tibiae. In metatarsals, osteoclasts must migrate before bone resorption can take place, whereas osteoclast migration is not a prerequisite for bone resorption in tibiae in these tissue cultures. BB-94 dose-dependently reduced the ^{45}Ca release in non-stimulated (A, B) and 10^{-8} M PTH-stimulated (C, D) metatarsals (A, C) and tibiae (B, D) cultured for seven days. The result (T/C) is the ratio between the active ^{45}Ca release in BB-94 treated bones and in paired vehicle treated controls. All values are Mean+SD (n=4).

Significance levels, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 18 shows the inhibitory effect of the hydroxamate-type MMP inhibitor, GM6001, on the decalcification of cultured metatarsals and tibiae. In metatarsals, osteoclasts must migrate before bone resorption can take place, whereas osteoclast migration is not a prerequisite for bone resorption in tibiae in these tissue cultures. GM6001 dose-dependently reduced the ^{45}Ca release in tibiae (white bars) and metatarsals (grey bars) cultured for seven days. The result (T/C) is the ratio between the active ^{45}Ca release in GM6001 treated bones and in paired vehicle treated controls. All values are Mean+SD (n=4).

Figure 19 shows the correlation between the individual IC_{50} values of a series of phosphinate based peptide derivatives and their K_i values against either MMP-7, MMP-9, MMP-12, MMP-13, MMP-14, or MMP-20.

Figure 20 shows the dose-dependent reduction by the novel phosphinate-inhibitor 01-07A of the 100 nM PTH-induced release of ^{45}Ca from cultures of pre-labelled foetal mouse calvariae. The effect at 10 µM 01-07A was similar to the effect of 10 µM GM6001. All values are Mean+SD (n=6).

Figure 21 shows the significantly ($p<0.05$) increased retention of ^{3}H-labelled tetracycline in tibiae and femurs isolated from mice treated *in vivo* with the MMP inhibitor RP59794 (200 µg s.c. bid) relative to vehicle (saline s.c. bid) treated littermates. Also the established inhibitors of bone resorption, clodronate (Cl2MBP), pamidronate (APD) and E-64 were efficient. All values are Mean+SD of respectively 4, 3, 4, 1 and 4 experiments each involving at least 5 mice per experimental group (RP59794, tibiae and femurs; clodronate, pamidronate, and E-64, tibiae).

Figure 22 shows the reduction in the development of osteolytic metastases in mice treated with the MMP inhibitor BB-94 at Day 19-28 after inoculation of human breast cancer cells (at day 0). The mice were X-rayed at Day 19 and those with osteolytic metastasis were randomised into two groups receiving either vehicle or BB-94 (60 mg/kg/day) by i.p. injection once daily until Day 28. There was no significantly difference in area of osteolytic lesions between the two groups before the treatment was initiated (white bars), and the area of osteolytic lesions was significantly increased (# $p < 0.00001$) for both group of mice at Day 28 (grey bars). However, the increase was significantly lower in BB-94 (*** $p < 0.005$). All values are Mean+SEM ($n = 9$, vehicle and $n = 12$, BB-94).

Figure 23 shows that the MMP inhibitor, GM6001 (100 mg/kg/day i.p. by single daily injections), was well tolerated by mice according to weight curves, and that it reduced the development of osteolytic metastases when given from Day -3 to 28 or Day -3 to 7 to mice inoculated with human breast cancer cells at Day 0. The increase in body weight was not significantly different between the three groups of animals (A). The area of osteolytic lesions was significantly suppressed (*: $p < 0.05$) in mice receiving GM6001 for the whole experimental period and also reduced in mice treated with GM6001 for just 11 days (B). All values are Mean+SEM ($n=7$).

Figure 24 shows the significantly increased survival (Wilcoxon rank t-test, $p < 0.0005$) of mice treated with the MMP inhibitor, GM6001 (100 mg/kg/day by single daily injection, "protease inhibitor") after cancer cell inoculation at Day 0. Three out of nine mice treated with GM6001 group survived until planned sacrifice at Day 49, whereas, all vehicle treated animals ($n = 9$) died within Day 36.

Figure 25 shows the significant reduction ($p<0.001$) in the number of MC3T3-E1 osteoblasts after 6 days of culture in 3-dimensional collagen gels induced by the hydroxamate-type MMP inhibitor, GM6001 (10 μM). In contrast, the inhibitors of serine proteinases, cysteine proteinases, and aspartic proteinases, i.e. aprotinin, E-64, and pepstatin (all 10 μM), respectively, did not affect the osteoblast number when compared to the vehicle-treated control. All values are Mean+SD (n=4) in each of 3 experiments.

Figure 26 shows the significant reduction ($p<0.001$) in the number of primary foetal mouse osteoblast after 6 days of culture in 3-dimensional collagen gels induced by the hydroxamate-type MMP inhibitors, GM6001 and BB-94 (10 μM). In contrast, the novel phosphinate-type MMP inhibitor, 01-07A did not affect the osteoblast number when compared to the vehicle-treated control. All values are Mean+SD (n=4) in each of 2 experiments.

Figure 27 shows the dose-dependent reduction in the number of MC3T3 osteoblastic cells when treated with the hydroxamate-type MMP inhibitor, GM6001, and the persistency of this osteoblast cell line when treated similarly with the novel phosphinate-type MMP inhibitor, 01-07A. All values are Mean+/-SD (n=4) in each of 2 experiments.

Figure 28 shows the dose dependent reduction of bone nodule formation by the hydroxamate-type MMP inhibitor, GM6001. The maximal effect was reached at 400 nM. All values are Mean+/-SD (n=4) in each of 2 experiments.

Figure 29 shows the significant reduction ($p<0.05$) in bone nodule formation by differentiated MC3T3-E1 cells when cultured in the presence of 10 μM GM6001. In contrast, the novel phosphinate-type MMP inhibitor, 01-07A did not affect the bone

formation when compared to the untreated control. All values are Mean+SD (n=4).

Compounds of the formula (I) in which R^1 and R^9 represent amino acids or peptides hereof are conveniently prepared by a solid phase peptide synthesis technique. Solid phase peptide synthesis has been standardised and peptides containing the phosphinic dipeptido-mimetic moiety (i.e., according to formula (I): -NH-CH(R^4)-P(X^a)(X^b-R^5)-C(R^6)(R^7)-CH(R^8)-CO-) were prepared using conventional protected amino acids in combination with a specially designed building block to incorporate the phosphinic acid moiety. The syntheses of the building blocks, **III**, were carried out in solution and in analogy to a literature report concerning similar building blocks (Yiotakis *et al*, 1996). As shown in Example 1, the selected Gly-Leu mimicking phosphinate building block had the following structure: Fmoc-Glyψ{P(O)(OAd)-CH_2}Leu-OH (i.e. **III** in which R^N = Fmoc, R^4 = R^6 = R^7 = H, X^a = X^b = O, R^5 = 1-Ad, R^8 = Bu^i). The structure of this building block was selected due to the fact that the Gly-Leu moiety is a preferred cleavage site for some MMPs in certain natural and synthetic substrates. We have found that both MMP-12 and MMP-14 readily cleave the extracellular matrix protein, osteopontin (OPN), at the particular Gly-Leu bond positioned between the amino acids Gly(159) and Leu(160) in OPN of bovine origin (boOPN): ...-Val-Ala-Tyr-Gly^{159}-^{160}Leu-Lys-Ser-Arg-... . Furthermore, the probably most widely used and very sensitive quenched fluorogenic synthetic peptide-like MMP-substrate, i.e. Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (M1895, Bachem, Switzerland) where Mca is (7-methoxycoumarin-4-yl)acetyl and Dpa is (2',4'-dinitrophenyl)-L-2,3-diaminopropionyl, is cleaved by MMPs at the Gly-Leu bond.

In order to study the influence of particular side chains in formula (I) on potency and selectivity towards various MMPs,

and with the expectation of thereby developing selective MMP inhibitors, several phosphinate based peptide derivatives were produced by solid phase peptide synthesis as shown in Example 2 and by using the aforementioned Gly-Leu mimicking phosphinate building block, Fmoc-Glyψ{P(O)(OAd)-CH_2}Leu-OH. Among these were the seven substrate-mimicking peptide derivatives, named 01-01 to 01-07, and the 22 PEGA bead inhibitory library derived peptide derivatives, 02-01 to 02-22. The building block was not resolved and consequently all peptide derivatives were produced in two diastereomeric forms (named the A- and B-forms), which were separated by reversed phase HPLC.

The phosphinate based peptide derivatives, 01-01 to 01-07 (see Table 4 of Example 9) were designed in analogy to the sequences of both natural bone-related protein substrates of MMPs and a small synthetic peptide substrate of MMPs in which the Gly-Leu (or Ala-Ile) moiety of the substrate cleavage site was replaced by the -Glyψ{P(O)(OH)-CH_2}Leu- moiety. The specific cleavage site of MMP-12 and MMP-14 that we have recently identified in boOPN: ...-Val-Ala-Tyr-Gly^{159}-^{160}Leu-Lys-Ser-Arg-... and the equal site: ...-Val-Val-Tyr-Gly^{167}-^{168}Leu-Arg-Ser-Lys-... in human OPN (huOPN), was used in the design of derivatives 01-01 to 01-03. The cleavage site of MMP-12 and -14 in bovine bone sialoprotein, boBSP: ...Gly-Leu-Ala-Ala^{134}-^{135}Ile-Trp-Leu-Pro-... and the equal site: ...Gly-Leu-Ala-Ala^{129}-^{130}Ile-Gln-Leu-Pro-... in human BSP (huBSP) was used in the design of derivatives 01-04 to 01-06. For the latter three derivatives, however, the inserted -Glyψ{P(O)(OH)-CH_2}Leu- moiety was expected to mimic the -Ala-Ile- moiety of the substrate cleavage site. Our formerly developed quenched fluorogenic peptide MMP-substrate, H-Abz-Gly-Pro-Leu-Gly-Leu-Tyr(NO_2)-Ala-Arg-OH (Renil *et al*, 1998), where Abz is the fluorogenic group, 2-aminobenzoic acid,

and Tyr(NO_2) is the fluorescence quencher, 3-nitro-tyrosine, was used for the design of derivative 01-07.

Instead of using a preformed phosphinic dipeptide building block (III), the phosphinic dipeptido-mimetic moiety may be formed directly during solid phase peptide synthesis, e.g. by reacting an N-terminally acryloylated resin-bound peptide, $C(R^6)(R^7)=CH(R^8)$-CO-peptide, with a protected 1-aminoalkylphosphinic acid, R^N-NH-$CH(R^4)$-$PH(X^a)(X^b$-H), in the presence of a trimethylsilylating agent. The acryloylated peptide can be obtained, e.g. by reacting the N-terminus of a peptide with a 2-alkylacrylic acid chloride. This alternative, direct approach for the solid phase synthesis of compounds of formula (I) is shown in Example 3 by synthesis of the phosphinate based peptide derivative, LMF-Alaψ{P(O)(OH)-CH_2}Gly-FAPFFG.

The presence of a starting peptide on the solid support is not essential. For instance an amino group may be provided on the solid support otherwise than by a peptide, e.g. by the use of a non-peptide photolabile linker. This can be used for: 1) making a combinatorial library of the form R-P2-P1(PC)P1′ (i.e. with just the P1′ position occupied in the C-terminal site) or 2) cleaving off the resulting phosphinate of the form: R1-NH-C(R2)(R3)-CO-NH-CHR4-P(Xa)(XbR5)-C(R6)(R7)-CH(R8)-C(O)(OH) (=R-P2-P1(PC)P1′.

Also, such methods of synthesis may in principle be practised in solution rather than using a solid support.

The phosphinate based peptide derivatives synthesised in the PEGA bead combinatorial library were of the general structure, X^1-X^2-X^3-Glyψ(PO_2-CH_2)Leu-X^4-X^5-X^6-, in which X^1 to X^6 represent amino acids. As shown in Example 4, it was constructed as a one-bead-two-compounds library, i.e. each bead contained a different phosphinate based peptide derivative and a quenched

fluorogenic substrate for MMPs which was common for all beads. During incubation of such a library with a metalloproteinase, e.g. MMP-9, MMP-12, MMP-13 or MMP-14, the substrate is either cleaved or it is not. For each bead, the amount of substrate cleaved is inversely proportional to the potency of its particular inhibitor. Because of the incorporation of the special donor-acceptor pair of intramolecular resonance energy transfer (Abz and Tyr(NO_2)), into the part of the substrate proximal and distal to the bead, respectively, it is possible by use of fluorescence microscopy to distinguish between beads containing substrate that has been cleaved and beads containing substrate that has not been cleaved to a considerable degree. In the beads containing substrate that has been cleaved to a considerable degree during incubation with the proteinase, the 3-nitrotyrosine will diffuse away from the 2-aminobenzoic acid, which will remain immobilised on the bead. Consequently, the bead will appear fluorescent when analysed by fluorescence microscopy. Thus, those beads that appear dark under a fluorescence microscope after incubation of the PEGA bead library with e.g., MMP-9, MMP-12, MMP-13 or MMP-14, contains a putative inhibitor and can be manually collected and sequenced.

As shown in Example 4, mixtures of Fmoc- and Boc-amino acids in the ratio 9:1 were used for each coupling cycle of the synthesis of the phosphinate based peptide derivatives in the library. Since only the Fmoc group was removed during the subsequent deprotection step, "a ladder" of fragments of all possible lengths with N-terminal Boc groups were present after the completion of the library synthesis. After removal of the acid labile protective groups (among these Boc), the PEGA bead library was incubated with proteinase. The ladder of fragments was released from each of the collected dark beads by cleavage of the light sensitive linker. This ladder-mixture consisted of

the full length phosphinate based peptide derivative and all possible fragments that were shorter because they did not include one or more of the N-terminal residues. The sequences of the mixture of phosphinate based peptide derivatives and peptides released from each dark bead were determined in a single MALDI-TOF mass spectrum. Each sequence was deduced directly from the differences between peaks of the mass spectrum. It should be noted that a drawback of this method is that the amino acids Leu, Ile and Hyp, as well as Lys and Gln have the same molecular weight and can therefore not be distinguished by this type of sequencing. For beads having one or more of these amino acids N-terminally to the phosphinate building block, conventional Edman degradation made it possible to distinguish between these residues.

The sequences present on active beads found by screening a solid phase combinatorial library with an MMP can be used directly for the design of soluble phosphinate based peptide derivatives of formula (I), which may then be analysed for their putative MMP inhibitory potential. Example 5 shows the sequences of 16 putative inhibitors (04-02 to 04-17) that were designed according to sequences determined by MALDI-TOF mass spectrum analysis of phosphinate based peptide derivatives positioned on beads that remained dark after incubation with MMP-12.

Furthermore, sequences present on the active beads found by screening a solid phase combinatorial library with an MMP can be analysed statistically to determine a consensus sequence that contains the most frequently occurring amino acids in each subsite. Frequently occurring combinations of two or more amino acids should also be noticed, since the subsites cannot be considered as completely independent. As shown in Example 6, incubation of a PEGA bead inhibitor library with MMP-12 resulted in the determination of putative inhibitor sequences from 82

dark beads, which were then used as a basis for establishment of the consensus sequence. The resulting consensus sequence as well as the sequences of 21 selected single amino acid substitutions in one of the positions corresponding to the P4 to P2 and the P2' to P4' positions were used for synthesis according to Example 2 of the derivatives 02-01 and 02-02 to 02-22, respectively. Furthermore, truncated forms of the consensus sequence 02-01 (04-01 and 04-21) and of 02-16 (04-20) and 02-22 (04-19) were designed for analysis of the importance of the P4, P3, P3', P4', and P5' positions. Finally, a "second generation" consensus sequence (04-18) was designed after kinetic analyses of 02-01 to 02-22. This sequence contained those amino acids, which for each subsite gave the most potent inhibitor in the previous single amino acid substitutions of the consensus sequence. This was mainly done in order to test for additivity of the effects of substitutions.

As an alternative to incubation of the PEGA bead inhibitor library with just a single MMP, sequential incubations with different proteinases can be used in order to search for selective inhibitors. Example 7 shows the experimental method, and the number of sequences obtained, by sequential incubations with MMP-9, MMP-13, and MMP-14 of a library analogous to the one used for incubation with MMP-12 alone (see above). The library was split into three portions for the first incubation with either MMP-9, MMP-13 or MMP-14. The resulting three portions of dark beads were separated from the fluorescent beads, and each split into two portions. Each of these two portions were incubated with a second MMP different from the MMP used in the first incubation. The resulting dark beads of the second incubation were incubated with the third MMP in a final incubation.

Phosphinate based peptide derivatives isolated from beads that were identified as fluorescent after either the second or later incubations with a proteinase but remained dark after the first or more incubations can be expected to be selective in their putative inhibitory activities. Example 8 shows the design of 40 sequences representing 10 of the 12 groups from the sequential incubations with MMP-9, MMP-13, and MMP-14 described in Example 7. The sequences were mainly based on the results from MALDI-TOF mass spectroscopy and used for the synthesis of soluble, putatively selective MMP inhibitors.

The kinetic characterisation of the soluble phosphinate based peptide derivatives of the present invention was primarily done in order to determine their K_i-values for a number of MMPs. A low K_i-value, preferably in the low or even subnanomolar range, shows that the inhibitor is potent against the particular MMP. The ratio between the K_i-value of an inhibitor for one particular MMP and the K_i-value of the same inhibitor for another MMP describes its selectivity. A ratio higher than 1 shows that the inhibitor is more potent against the second MMP, and a ratio below 1 shows that the inhibitor is more potent against the first inhibitor. The K_i-values of the 7 substrate-mimicking phosphinate based peptide derivatives (01-01 to 01-07) in both their A and B diastereomeric forms are described for MMP-1, -3, -9, -12, and -14 in Example 9. Neither the 3 inhibitor sequences (01-01 to 01-03) mimicking the MMP-12 cleavage site in boOPN or huOPN, nor the 3 inhibitor sequences (01-04 to 01-06) mimicking the MMP-12 cleavage site in boBSP or huBSP, had very low K_i-values for the MMPs tested. In contrast, the inhibitor 01-07 with the sequence, Ala-Gly-Pro-Leu-Glyψ{PO_2-CH_2}Leu-Tyr-Ala-Arg-Gly, mimicking a sensitive quenched fluorogenic synthetic MMP-substrate, was very potent against MMP-2, MMP-9, and MMP-13 (K_i 1.2 to 2.2 nM for the A-

form) and also moderately strong against MMP-1, MMP-7, MMP-14, and MMP-20 (10 to 75 nM), but weak towards MMP-12 (200 nM) and MMP-3 (2,200 nM). This phosphinate based peptide derivative therefore represents a potent and somewhat selective inhibitor for MMP-9. MMP-9 is one of the most abundant proteinases in osteoclasts and probably plays a major role in osteoclast invasion and migration, and 01-07A therefore could be an important regulator of bone metabolism.

The soluble phosphinate based peptide derivative (03-01), which was synthesized by the direct approach (see Example 3) and contains -Alaψ{P(O)(OH)-CH_2}Gly- at its P1-P1´ position was mainly prepared for documentation of the alternative route of synthesis and for comparison to phosphinate based inhibitors of the -Glyψ{P(O)(OH)-CH_2}Leu- containing type. As shown in Example 9, and as expected, 03-01A was not a very efficient MMP-inhibitor according to kinetic analyses with MMP-9, MMP-12, MMP-13 and MMP-20 (all K_i values above 10 μM).

The sixteen phosphinate based peptide derivatives (04-02 to 04-17), which were designed directly according to sequences found on active beads from a solid phase combinatorial library incubated with MMP-12 (see Example 5), were synthesised in soluble form according to Example 2. As shown in Example 10 the MMP-12 inhibitory activities of thirteen of these phosphinic peptides (04-03A to 04-05A, 04-07A, and 04-09A to 04-17A) were confirmed, showing K_i values of 1 μM or less. Considering that the K_m value for the substrate used in the library is in the low μM range, all beads containing phosphinic peptides with Ki values of 1 μM or less will appear as dark (positive) beads. In three of the phosphinic peptides (04-02A, 04-06A, and 04-08A) the MMP-12 inhibitory activity could not be confirmed by enzymatic assay.

Twenty-two phosphinate based peptide derivatives (02-01 to 02-22), synthesised according to the consensus sequence determined from the result obtained by incubation of the X^1-X^2-X^3-Glyψ(PO_2-CH_2)Leu-X^4-X^5-X^6-designed PEGA-bead library with MMP-12, were characterised by kinetic analyses. As shown in Example 10, most of them were, in their respective diastereomeric A-form, potent inhibitors of MMP-12 (K_i-values in the low nanomolar range) and some of them (e.g., 02-13A and 02-16A) were very selective according to comparative analyses with MMP-9 and MMP-13 (K_i-values in the micromolar range), whereas others (e.g., 02-06A and 02-07A) were almost as, or even more, potent for MMP-9 and/or MMP-13. Thus, both selective and non-selective inhibitors of MMP-12 were developed, and since MMP-12 appears to be an MMP which is mainly present in cells of the macrophage/osteoclast lineage, where it is hypothesised to play a role in invasion, these inhibitors are promising compounds for regulation of bone metabolism.

Of the 40 soluble phosphinate based peptide derivatives representing 10 of the 12 possible combinations of three sequential incubations of the PEGA bead inhibitor library with MMP-9, MMP-13, and MMP-14 groups, just a minor fraction reacted as expected when undergoing kinetic analysis with the relevant MMPs. As shown in Example 11, several of the synthesized phosphinic peptides had K_i values in the nanomolar range, but their pattern of reaction with MMP-9, MMP-13, and MMP-14, more often than not was unlike the pattern of reactivities that was observed for the corresponding bead. Some of the surprising results may have been due to the lack of purification of the 40 compounds resulting in testing of the mixed distereomeric form (A/B) rather than the individual A- (and B-)form(s). Still, some interesting selective MMP-

inhibitors have been identified already by use of this new method, and more may be obtained through continuous screening among the total of 131 to 208 sequences identified by the sequential incubations or by repeating the sequential incubations under more fierce conditions using a freshly prepared combinatorial library.

We have previously shown that natural as well as synthetic hydroxamate-type MMP inhibitors are able to inhibit the invasion of osteoclasts through a type I collagen matrix, thereby reducing bone resorption *in vitro* (Sato *et al*, 1998). In contrast, inhibitors of serine, cysteine and aspartic proteinases did not inhibit osteoclast invasion through collagen (Sato *et al*, 1998). The analyses described in Example 12 show that the novel phosphinate based peptide derivatives are functional MMP inhibitors under biological test conditions, that they can regulate osteoclast invasion through type I collagen, and that their effect on osteoclast invasion is proportional to their potency towards MMP-9 determined by kinetic analyses. Thus, the novel phosphinate-type MMP inhibitors, such as 01-07A, appear to be promising compounds for regulation of osteoclast invasion.

The recruitment of osteoclasts to their future site of resorption requires motility of the osteoclast and therefore also interaction with the extracellular matrix. We have observed that not only when invading through a type I collagen layer, but also when migrating over a type I collagen surface, MMPs appear to be of central importance for collagen degradation and osteoclast motility. In Example 13, the collagenolysis observed in the vicinity of individual osteoclasts was correlated to their distance of migration, and it was shown that inhibition of the MMP-activity under these conditions does reduce both degradation of type I collagen and

osteoclast migration. Thus, MMP-inhibition appears to be a suitable way to regulate osteoclasts migration.

When osteoclasts migrate to their future site of resorption they must usually pass through a shielding layer of bone lining osteoblasts. In order to mimic the natural situation, we have developed a model which includes a confluent bone lining cell layer protecting the bone surface from degrading osteoclast. As shown in Example 14, inhibition of the MMP-activity during culture of osteoclasts seeded on this layer of bone lining cells reduced bone resorption. Thus, MMP-inhibition appears to be a suitable way to regulate osteoclasts invasion through the shielding layer of bone lining cells.

The efficacy of the bone lining cell barrier to osteoclast passage is regulated by certain hormones and growth factors, including PTH and TGF-β, respectively. Bone lining cells retract quickly and intermittently when exposed to PTH (or PTHrP), which in part could explain the induction of bone resorption by this hormone. We have found that also TGF-β induces bone resorption by regulation of the access of osteoclasts to the bone surface through the layer of bone lining cells. However, the morphological change of bone lining cells induced by TGF-β is different from that of PTH. The cells undergo a contracting elongation by intracellular actin rearrangement and thereby expose some of the otherwise covered bone surface. When studied in cultures of osteoblasts, the maximum accessible surface appears already after 1 hour of exposure to PTH, but after 24 hours of exposure to TGF-β. In contrast, recovery of the bone lining cell layer takes place within 1 day of PTH exposure, even if PTH is added continuously, whereas the recovery after TGF-β exposure is much slower and will not happen as long as the growth factor is

still present. However, we have found that the regulation by TGF-β of the morphology of bone lining cells depends on MMP activity.

As shown in Example 15, the 2-3 fold increase in accessible surface area which was induced by TGF-β treatment of the bone lining cell layer was significantly reduced by MMP-inhibition, but not by inhibition of serine, cysteine or aspartic proteinases. Furthermore, this reduction was as strong for the MMP-9 selective phosphinate based peptide derivative, 01-07A, as it was for two potent general MMP inhibitors of the hydroxamate-type. When the bone lining cell layer was instead established on slices of a bone substratum, the treatment with TGF-β resulted, as expected, in a reduced resistance to subsequent osteoclast invasion and thereby to increased bone resorption. However, simultaneous treatment with an MMP inhibitor counteracted the TGF-β stimulation and reduced bone resorption. Thus, MMP-inhibition appears to be a suitable way to regulate the growth factor controlled bone lining cell barrier to osteoclast access.

In contrast to analyses of MMP-inhibition in bone cell cultures, the use of bone tissue cultures allows the study of complex interaction between many cell types which are spatially and temporally organised as under *in vivo* conditions. When the experiments of Example 15 were extended to include cultures of calvariae which were treated in the same way as the bone lining cell cultures, i.e. with TGF-β in the absence or presence of an MMP inhibitor, such as the phosphinate based peptide derivative, 01-07A, they responded similarly. Thus, bone resorption in the calvariae was increased by TGF-β, but the increase was abolished by simultaneous treatment with the novel phosphinate-type MMP inhibitor, 01-07A.

An established tissue culture method for characterisation of the interactive processes of osteoclast recruitment, invasion and bone resorption in foetal mouse metatarsal and tibia cultures was used in order to characterise the effects of phosphinate based peptide derivatives. At this stage in foetal development, mononuclear osteoclasts are confined to the periosteum surrounding the newly calcified cartilage in metatarsals. During *in vivo* development as well as in culture, these osteoclasts mature, invade and resorb the calcified cartilage forming a primitive bone marrow cavity in the metatarsals. In contrast, mature bone resorbing osteoclasts are already present in the primitive bone marrow cavity of tibiae of the 17-day-old mouse foetuses. Thus, the bone resorption measured in tibia cultures reflects predominantly resorptive activity *per se* of mature osteoclasts and to a lesser extent their maturation and invasion. As shown in Example 16, the two potent, hydroxamate-type MMP inhibitors, BB-94 and GM6001, which have low or sub-nanomolar K_i-values and little selectivity towards a variety of MMPs, including MMP-1, -2, -3, -7, -9, -12, -13, -14, and -20, as well as several non-MMP metalloproteinases, including some of the ADAMs, not only inhibited the recruitment and migration but also reduced the resorptive activity of osteoclasts in bone cultures. In contrast, the potent MMP-9 selective phosphinate-type inhibitor, 01-07A (see Table 4 of Example 9), selectively inhibited the recruitment and migration but did not affect the resorptive activity of osteoclasts. Thus, the selectivity for MMP-9 of some of the novel phosphinate-type MMP inhibitors, such as 01-07A, appears to confer a specific effect on osteoclast recruitment and migration. This finding is strongly supported by our recent investigations of metatarsals and tibiae isolated from transgenic mice specifically deficient

for the MMP-9 gene (i.e., so-called MMP-9 knockout mice) (Chen *et al*, 1998). When male and female mice heterozygous for the functional MMP-9 gene were mated, approx. 25% of the foetuses in a litter were MMP-9 knockouts. Metatarsals isolated from 17-day-old foetuses that were MMP-9 knockouts had an approx. 50% reduction in decalcification during culture compared to their heterozygous and wild-type littermates. When these metatarsals were cultured in the presence of an MMP inhibitor, the decalcification was completely blocked no matter whether the bones were isolated from foetuses with a functional MMP-9 gene or not. Furthermore, an impeded and delayed recruitment and invasion of osteoclasts into the central part of freshly isolated metatarsals from MMP-9 knockout mouse foetuses was observed by histological inspections. In contrast, the decalcification in cultured tibiae was similar in bones from MMP-9 knockout foetuses and littermates with a functional MMP-9 gene. Thus, an MMP-9 selective inhibitor such as 01-07A which act specifically on osteoclast recruitment and invasion both in invasion assays based on osteoclast and bone lining cell cultures as described in Examples 12 and 15, respectively, and in the metatarsal assay based bone tissue cultures described in Example 16, is likely to have the same effect under *in vivo* conditions.

It is commonly believed that cathepsin K is the most important proteinase used by osteoclasts to solubilise the bone matrix in the subosteoclastic resorption zone. However, it was shown recently that the levels of cathepsin K in osteoclasts of calvariae are low compared to those of long bones (Everts *et al*, 1999). Accordingly, calvariae of cathepsin K knockout mice have no apparent phenotype, whereas their long bones show deficient resorption (Saftig *et al*, 1998). These observations raise the question of what

proteinases are then used by the osteoclast to solubilise the bone matrix in calvariae. As shown by the quantitative ultrastructural studies of demineralised collagen fibres in the subosteoclastic resorption zone of PTH-stimulated calvaria cultures described in Example 17, not only cysteine proteinases but also MMPs play a rate limiting role in the solubilisation of bone matrix by osteoclasts. In the same cultures, it was also interesting to notice that the areas of demineralised matrix in those resorption pits from which the osteoclasts had disappeared were much larger when the calvariae were cultured in the presence of an MMP inhibitor than when they were cultured with a cysteine proteinase inhibitor. The ultrastructural inspections described in Example 17 showed that after the osteoclast had left its resorption pit, bone lining cells occupied this pit and exhibited phagocytic activity towards the collagen remnants left by the osteoclast. In the absence of a proteinase inhibitor or in the presence of a cysteine proteinase inhibitor, the areas of collagen remnants dropped to 20% of the initial values due to the lining cell proteolysis, whereas this collagen degradation was almost completely blocked by MMP-inhibition. These observations show (1) that in situations where osteoclastic collagenolysis is inhibited, bone resorption proceeds in 2-steps: the osteoclasts first demineralise the bones, and the bone lining cells then degrade the demineralised collagen; and (2) that MMP inhibitors can efficiently inhibit the latter process, whereas cysteine proteinase inhibitors cannot.

As shown in Example 18, the regulation of bone resorption in calvariae by MMP inhibitors depended on whether the process was stimulated or not. Whereas there was no effect of either a hydroxamate-type MMP inhibitor or the phosphinate based peptide derivative, 01-07A, on the ^{45}Ca release from non-stimulated

calvariae, both compounds almost completely inhibited the increase in bone resorption induced by 100 nM PTH. Thus, novel phosphinate-type MMP inhibitors, such as 01-07A, may be suitable for reduction of stimulated bone resorption.

As indicated above, there is strong evidence supporting a role of MMPs in bone resorption, but this evidence is largely based on bone cell and tissue culture models that, by definition do not precisely mimic *in vivo* situations. As shown in Example 19, we have developed an experimental model allowing the evaluation of the *in vivo* effect of MMP inhibitors and other compounds on bone resorption in newborn mice. The model is based on observations showing that bone resorption rates can be assessed by measuring ^{3}H-tetracycline retention in prelabelled bones (Klein *et al*, 1990). Tibiae and femurs of mice treated with the hydroxamate-type MMP inhibitor, RP59794, had significantly elevated contents of ^{3}H compared to the corresponding bones of non-treated control group of mice, and as high levels of ^{3}H as bisphosphonate and cysteine proteinase inhibitor treated groups. Thus, MMP-inhibition appears to be a suitable way to inhibit bone resorption *in vivo*.

Acute and local pathological bone metabolism is observed in patients with bone metastasis, and, as shown in Example 20, also in an experimental model of bone metastasis, which is based on intracardial inoculation of human breast cancer cells into nude mice. In this model, the cancer cells metastasise to bone and induce osteoclast-dependent osteolysis. A significant reduction in the number and size of osteolytic metastases was observed when mice inoculated with breast cancer cells were treated with an MMP-inhibitor either after radiographical demonstration of osteolytic lesions, or continuously from around the time of inoculation. According to daily inspections of the animals, the general condition of the MMP inhibitor treated mice

appeared normal as for the vehicle treated control mice until approx. 3 weeks after cancer cell inoculation and then was clearly better than in the control due to reduced or lack of cachexia. Furthermore, the survival was significantly prolonged for mice treated with MMP inhibitor. The major effect of the MMP inhibitor seemed to be a reduction of osteoclast recruitment and bone resorption activity, which restricted the expansion of the metastatic areas. Thus, MMP-inhibition appears to be a suitable way to both prevent the development of bone metastases and treat manifested osteolytic metastases.

The use of an MMP inhibitor for treatment of a particular disease or disorder introduces an inevitable risk of side effects, particularly due to the fact that most MMPs have a multitude of physiological functions in many tissues. Furthermore, most potent synthetic MMP inhibitors are strong inhibitors of several, if not all, members of the MMP-family as well as of several other related metalloproteinases such as the ADAMs (i.e., proteinases belonging to the "A Disintegrin And Metalloproteinase" family). There are several ways to reduce the potential side effects of MMP inhibitors, including the use of local administration near the site(s) of pathological excessive MMP activity, and the use of a restrictive treatment regimen, e.g. through intermittent dosing. However, a more efficient way to reduce the side effects is to improve the selectivity of the MMP inhibitor without compromising its potency towards the MMPs involved in pathogenesis. Phosphinate based peptide derivatives spanning both the P- and P'-corresponding sides are particularly suitable for optimisation of selectivity, since they can interact with both the S- and S'-pockets of the target MMP(s), and become repelled by non-target proteinases due to one or more of the amino acid or amino acid-mimicking residues of the pseudopeptidic compound being unable to dock and/or fit into the

catalytic site. For phosphinate based peptide derivatives which are developed in order to act as regulators of bone metabolism, it seems particularly important that their inhibition is selective for MMP(s) which are directly or indirectly involved in catabolic bone processes, in contrast to MMP(s) directly or indirectly involved in anabolic bone processes. Thus, certain MMPs of osteoclast origin, such as MMP-9 and MMP-14, which appears to be involved in osteoclast recruitment and invasion, as well as some MMPs of tumour cell or osteoblast origin, such as MMP-2 and MMP-13, which may be involved in upregulation of bone resorption, are preferred targets for selective inhibition. As shown in Examples 21 and 22, two potent hydroxamate-type MMP inhibitors which have low or sub-nanomolar K_i-values and little selectivity towards a variety of MMPs, including MMP-1, -2, -3, -7, -9, -12, -13, -14, and -20, as well as several non-MMP metalloproteinases, including some of the ADAMs, have an adverse effect on the number of osteoblasts and also reduce the bone formation by osteoblasts *in vitro*. These side effects were not observed for the phosphinate-type MMP-9 selective inhibitor, 01-07A. Thus, treatment with selective MMP inhibitors, such as 01-07A, will be less prone to cause severe side effects than MMP inhibitors which act on many different proteinases.

Further detailed description of methods suitable for use in this invention appear in Buchardt et al, "Phosphinic Peptide Matrix Metalloproteinase -9 Inhibitors by Solid-Phase Synthesis Using a Building Block Approach', published after the priority date hereof, Chem. Eur. J. 1999, 5, No.10, 2877-2884.

Example 1

Synthesis of the *O*-Adamantyl *P*-(9-fluorenylmethyloxy-carbonyl-aminomethyl)-*P*-(2-isobutylpropionic acid-3-yl)phosphinate, building block, **III** (R^N = Fmoc, $R^4 = R^6 = R^7$ = H, $X^a = X^b$ = O, R^5 = 1-Ad, $R^8 = Bu^i$, re: formula (**III**) in Fig. 1).

See also the reaction scheme in Fig. 1.

Step A:

Synthesis of *P*-(benzyloxycarbonylaminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)phosphinic acid (**IV**, R^N = Cbz, $R^4 = R^6 = R^7$ = H, $R^8 = Bu^i$, R^C = Et):

A mixture of benzyloxycarbonyl 1-aminomethyl phosphinic acid (5.00 g, 21.8 mmol) (Baylis *et al*, 1984) and hexamethyldisilazane (23 ml, 109 mmol, 5 eq.) was stirred under Ar in a dried 250 ml round-bottomed flask at 115°C for a period of 2 hours after which the temperature was lowered to 95°C over a period of 35 min. Ethyl α-isobutylacrylate (4.76 ml, 28.3 mmol, 1.3 eq.) was added dropwise to the opaque mixture in 35 min. After stirring for 3.5 h the mixture was clear and colourless. The temperature was lowered to 70°C and EtOH (65 ml) was carefully added. Cooling to room temperature and subsequent concentration *in vacuo* yielded a white solid which was dissolved in ethyl acetate (50 ml) and washed with 1 M HCl (50 ml). The aqueous phase was extracted with ethyl acetate (3x25 ml) and the combined extracts were washed with water (2x50 ml), saturated brine (50 ml), dried with Na_2SO_4 and concentrated dryness at high vacuum to give a white solid. Yield: 8.03 g (96%).

Step B:

O-Adamantyl *P*-(benzyloxycarbonyl-aminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)phosphinate (**V**, R^N = Cbz, R^4 = R^6 = R^7 = H, R^5 = 1-Ad, R^8 = Bu^i, R^C = Et):

A mixture of *P*-(benzyloxycarbonylaminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)phosphinic acid (**IV**, 820 mg, 2.12 mmol) and Ag_2O (986 mg, 4.25 mmol, 2 eq.) was refluxed under Ar in anhydrous chloroform (3 ml) for 15 min after which a chloroform solution of AdBr (503 mg, 2.34 mmol, 1.1 eq.) was added dropwise to the refluxing suspension over a period of 30 min. Reflux was continued for 1 h and the mixture was stirred overnight. The crude mixture was filtered through Celite, concentrated and purified by vacuum liquid chromatography using toluene:ethyl acetate 3:1 as eluent to obtain a highly viscous syrup. Yield: 977 mg (89%).

Step C:

O-Adamantyl *P*-(9-fluorenylmethyloxycarbonyl-aminomethyl)-*P*-(2-isobutylpropionic acid-3-yl)phosphinate (**III**, R^N= Fmoc, R^4= R^6= R^7= H, R^5= 1-Ad, R^8= Bu^i):

Aqueous NaOH (11.8 ml, 4M, 47.2 mmol, 4 eq.) was added dropwise to a solution of *O*-Adamantyl *P*-(benzyloxycarbonyl-aminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)phosphinate (**V**, 5.55 g, 11.8 mmol) in EtOH (100 ml). The resulting opaque solution was stirred for 24 h after which it was concentrated, the residue mixed with ethyl acetate (100 ml) and water (50 ml), cooled to 0°C and HCl (aq., 20 ml 2.4 M, then 8 ml 1 M) was slowly added to adjust pH to 2. The aqueous phase was extracted twice with ethyl acetate (50 ml), the combined extracts were dried with $MgSO_4$ and concentrated to dryness, affording a white solid carboxylic acid. The crude carboxylic acid (200 mg, 407 µmol) was dissolved in ethyl

acetate+MeOH+water (11+6+0.5 ml) and hydrogenated at atmospheric pressure in the presence of Pd (5% on activated carbon, 86 mg, 41 µmol, 0.1 eq.), $NaHCO_3$ (171 mg, 2.0 mmol, 5 eq.) and Fmoc-OSu (206 mg, 610 mmol, 1.5 eq.) for a period of 80 min. Vacuum (10 mmHg) was then applied for 30 min and the mixture was left stirring overnight. Filtration through Celite and concentration gave the crude product as a sticky solid (411 mg) which was purified by vacuum liquid chromatography using chloroform:methanol 30:1 as eluent to give a solid foam. Yield: 154 mg (65%).

Example 2

Synthesis of compounds of formula (I) by solid phase synthesis, exemplified by synthesis of the phosphinate based peptide derivative, H-Ala-Gly-Pro-Leu-GlyΨ{PO_2H-CH_2}Leu-Tyr-Ala-Arg-Gly-OH (01-07) using the building block (III).

See also the general reaction scheme in Fig. 2.

Step A:

A pre-activated (10 min) mixture of hydroxymethylbenzoic acid (657 mg, 4.32 mmol, 3 eq.), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (1.33 g, 4.15 mmol, 2.88 eq.) and *N*-ethylmorpholine (727 µl, 662 mg, 5.76 mmol, 4 eq.) in anhydrous DMF (25 ml) was added to the resin ($PEGA_{800}$-resin, 0.48 µmol/mg, 3.01 g, 1.44 mmol) swelled in DMF. After 2 h the resin was washed with DMF, dichloromethane and lyophilised overnight.

Step B:

The resin was swelled in anhydrous dichloromethane (40 ml) and Fmoc-G-OH was coupled using 2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazolide (2 couplings, 65 min and 75 min) (Blankemeyer-Menge *et al, 1990)*: Fmoc-G-OH (1.28 g, 4.32 mmol, 3 eq.) was dissolved in dry DCM (20 ml) together with *N*-methyl imidazole (258 µl, 266 mg, 3.24 mmol, 2.25 eq.) and when dissolved 2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazolide (1.28 g, 4.32 mmol, 3 eq.) was added. The mixture was immediately added to the resin. After the second reaction the resin was washed with DCM, lyophilised and 75 mg (36 µmol) of the resin was weighed out for further synthesis. Couplings of the amino acids Arg(Pmc), Ala and Tyr(Bu^t) was carried out using α*N*-Fmoc protected amino acid pentafluorophenyl esters (3 eq.) in anhydrous DMF and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (1 eq.) as catalyst. After coupling of an amino acid the resin was washed with DMF, followed by removal of the Fmoc group by treatment with piperidine (20% in DMF) for 2 and 10 min. After washing with DMF the cycle was repeated with the next amino acid.

Step C:

Coupling of the building block **III** (R^N= Fmoc, R^4= R^6= R^7= H, R^5= 1-Ad, R^8= Bu^i) (1.5 eq., 31.3 mg) was accomplished by activation with *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyl-uronium tetrafluoroborate (1.44 eq.) as above (Step A), and was completed in 4.5 h.

Step D:

After attachment of the building block, Fmoc was removed and Leu, Pro, Gly and Ala was attached by peptide synthesis as described above for Arg(Pmc), Ala and Tyr(Bu^t) (Step B). The

resin was then washed with DMF and DCM, dried by lyophilisation for 1.5 h and treated with a cocktail composed of trifluoroacetic acid:dichloromethane:H_2O: MeSPh: $(CH_2SH)_2$:triisopropylsilane 66.5:20:5:5:2.5:1 for 2.5 h to remove peptide side-chain protective groups. The resin was washed with AcOH, DMF, diisopropylethyl amine (5% in DMF), DMF, dichloromethane and lyophilized (1 h).

Step E:

The deprotected peptide was cleaved from the support by treatment with NaOH (0.1 M, 2x1.5 ml each, 1 h) and the resin was washed with H_2O (0.5 ml) and MeOH (0.5 ml). The resulting peptide solution (~5 mM) was neutralised with HCl (0.1 M, 2.5 ml), lyophilised and purified by reversed phase HPLC. Yield: 12 mg (35%), split between two diastereomeric compounds.

Example 3

Synthesis of compounds of formula (I) by solid phase synthesis using a direct approach, exemplified by the synthesis of the phosphinate based peptide derivative, H-Leu-Met-Phe-Alaψ{PO_2H-CH_2}Gly-Phe-Ala-Pro-Phe-Phe-Gly-OH (03-01)

See also the general reaction scheme in Fig. 3.

Step A:

POEPS-3 resin (1.5 g, 0.3 mmol) (Buchardt and Meldal, 1998) was swelled in anhydrous DCM (50 ml) and drained after a period of 10 min. A solution of Fmoc-Gly-OH (267 mg, 0.9 mmol) and N-methylimidazole (54 µl, 0.675 mmol) in anhydrous DCM (30 ml) was mixed with 2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazolide (267 mg, 0.9 mmol) and added to the resin. After 60

min the resin was drained and washed with anhydrous DCM x 3 and another coupling of Fmoc-Gly-OH/2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazolide/N-methylimidazole was performed for 60 min. The resin was drained and washed with DCM, DMF x 3, (5% diisopropylethylamine in DMF) x 2, DMF x 3, DCM and lyophilized. Loading: 0.20 mmol/g. A fraction of the resin (840 mg, 167 μmol) was treated with piperidine to remove Fmoc, washed with DMF, and treated with a solution of Fmoc-Phe-OPfp (278 mg, 501 μmol) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (27 mg, 167 μmol) in DMF for 1 h. Similarly, Fmoc protected amino acid Pfp esters of Phe, Pro, Ala, and Phe were coupled using 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine until a negative Kaiser ninhydrin test was observed (amounts, Fmoc-Pro-OPfp: 253 mg, Fmoc-Ala-OPfp: 240 mg). For the particular example, the product of this step was: Fmoc-FAPFFG-O-[POEPS-3 resin].

Step B

A fraction of the resin (200 mg, 40 μmol) was treated with piperidine and washed with DMF and DCM. Neat Et_3N (111 μl, 800 μmol) was added to the resin followed by a solution of the glycine analogue, acryloyl chloride (33 μl, 400 μmol) in DCM. The reaction was complete within 5 min according to Kaiser ninhydrin test. The resin was drained, washed with DCM and MeOH x 3, and lyophilised. For the particular example, the product of this step was: CH_2=CH-CO-FAPFFG-O-[POEPS-3 resin].

Step C

A fraction of the resin (50 mg, 10 μmol) was placed in a 5 ml reaction vial and a solution of the alanine analogue, 1-(allyloxycarbonylamino)ethylphosphinic acid (23.2 mg, 120 μ

mol) and bis(trimethylsilyl)-acetamide (90 µl, 360 µmol) in degassed 1,2-dichloroethane (1 ml) was added to the resin. The resin slurry was purged with Ar for 1 min, the vial was closed and heated to 100°C for 4 h after which the resin was cooled to room temperature and washed with DCM and lyophilised. For the particular example, the product of this step was: Alloc-NH-CH(CH_3)-P(O)(OH)-CH_2-CH_2-CO-FAPFFG-O-[POEPS-3 resin], or in short: Alloc-Alaψ{PO_2H-CH_2}Gly-FAPFFG-O-[POEPS-3 resin].

Step D

A solution of $Pd(PPh_3)_4$ (32 mg, 30 µmol) in $CHCl_3$:AcOH:N-ethylmorpholine 92.5:5:2.5 (800 µl) was added to the resin and left for 15 min. The resin was washed with $CHCl_3$, DMF x 3, (0.5% Et_2NCS_2Na in DMF) x 3 and DMF. A solution of Fmoc-Phe-OPfp (33 mg, 60 µmol) and Dhbt-OH (0.6 mg, 10 µmol) in DMF was added to the resin and allowed to react for a period of 16 h. Similarly, Fmoc protected amino acid Pfp esters of Met and Leu were coupled using 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (amounts Met: 16 mg, 30 µmol and Leu: 16 mg, 30 µmol). After the final coupling the resin was washed with DMF, treated with piperidine to remove Fmoc and washed with DMF and MeOH. For the particular example, the product of this step was: H-LMF-Alaψ{PO_2H-CH_2}Gly-FAPFFG-O-[POEPS-3 resin].

Step E

The product was cleaved from the resin by treatment with NaOH (400 µl, 0.1 M), the resin washed with water and MeOH. Aqueous HCl (400 µl, 0.1 M) was added to the combined filtrate and the solvents were removed in vacuo. The solid residue (10 mg) was redissolved in 50 % aqeous MeCN (2 ml) and purified by

preparative reversed phase HPLC. Fractions containing the product were collected and concentrated to dryness in vacuo to obtain, for the particular example, the phosphinic undecapeptide H-Leu-Met-Phe-Alaψ{PO_2H-CH_2}Gly-Phe-Ala-Pro-Phe-Phe-Gly-OH as a solid mixture of two diastereomers (5 mg, 45 % based on the initial resin loading or 90 % based on loading of Fmoc-FAPFFG-O-[POEPS-3 resin]).

Example 4

Preparation and screening of a one-bead-two-compounds solid phase combinatorial library of phosphinic peptides.

Synthesis of the library (see also the reaction scheme in Fig. 4).

Step A:

A solution of Fmoc-Lys(Alloc)-OH (2 eq., 119.5 mg), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (1.92 eq, 81.4 mg) and *N*-ethylmorpholine (2.67 eq., 44.5 µl) in anhydrous DMF was added to $PEGA_{1900}$-resin (0.11 mmol/g, 1.20 g, 0.132 mmol) and allowed to react for a period of 3.5 h. The resin was washed with DMF, Fmoc was removed (see Example 2), and the resin washed with DMF. A solution of Fmoc-Lys(Boc)-OPfp (3 eq., 251.5 mg) in anhydrous DMF was added to the resin and after a period of 1 h reaction was complete. The resin was washed with DMF, Fmoc was removed, and the resin was washed with DMF. The resin was treated with a solution of 3,4-dihydro-3-acetoxy-4-oxo-1,2,3-benzotriazine (1.5 eq., 40.6 mg) in anhydrous DMF for a period of 18 h. It was washed with DMF, dichloromethane, and treated with trifluoroacetic acid: water

19:1 for a period of 1 h after which it was washed with AcOH, dichloromethane, DMF, 5% diisopropylethylamine in DMF and DMF.

Step B:

The photolabile linker (3 eq., 206 mg) (Holmes and Jones, 1995) was coupled using *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetra-fluoroborate (2.88 eq, 122 mg) and *N*-ethylmorpholine (4 eq., 67 µl) in anhydrous DMF and coupling was complete in 17 h. All resin manipulations beyond this point were carried out avoiding direct ceiling- and sunlight, and reaction vessels were wrapped in aluminium foil. Fmoc was removed and after wash with DMF the amino acids Ile, Thr(Bu^t), Arg(Pmc), Ser(Bu^t), and Ile were attached to resin using Fmoc amino acid pentafluorophenyl esters (3 eq., see Example 2) and afterwards Thr (a mixture of 2.7 eq. Fmoc-Thr(Bu^t)-OH and 0.3 eq. Boc-Thr(Bu^t)-OH) was coupled using *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyl-uronium tetrafluoroborate (2.88 eq, 122 mg) and *N*-ethylmorpholine (4 eq., 67 µl). The resin was washed with DMF and dichloromethane and lyophilised. After reswelling the resin in DMF it was placed in a 20 well multiple column peptide synthesiser together with excess DMF and distributed equally between the wells.

Steps C and D:

The combinatorial synthesis was carried out by coupling 9:1 mixtures of 20 different Fmoc- and Boc-amino acids (Ala, Arg(Pmc), Asn(Trt), Asp(Bu^t), Gln(Trt), Glu(Bu^t), Gly, His(Trt), Ile, Leu, Lys(Boc), Met, Phe, Pro, Ser(Bu^t), Thr(Bu^t), Tyr(Bu^t), Val, Hyp and Hyl(Boc)) by the cyclic protocol below.

1. Coupling of amino acids. Each amino acid in a specific well. Stock solutions of Fmoc- + Boc-amino acids were made and

before each coupling 250 µl DMF was added to each well to avoid leaking from the wells. Mixtures of amino acids, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate and *N*-ethylmorpholine was then added to the resin.

2. Wash with DMF.

3. Mixing the resin. Excess DMF was added to the synthesiser, which was capped with the lid and shaken thoroughly for 1 h.

4. Removal of Fmoc (see Example 2).

5. Wash with DMF.

6. Repeat from 1.

After three couplings the building block **III** (R^N= Fmoc, R^4= R^6= R^7= H, R^5= 1-Ad, R^8= Bu^i) was coupled (as described in Example 2) and three more combinatorial couplings were performed by the above protocol. After the last coupling the resin was transferred to a flat-bottomed syringe with Teflon fritte and washed with DMF, split into three equal portions which were washed with dichloromethane and lyophilised.

Step E:

One third of the resin was treated with $Pd(PPh_3)_4$ (3 eq., 139 mg) in a degassed mixture of chloroform:AcOH:*N*-ethylmorpholine 92.5:5:2.5 for a period of 4.5 h and washed with chloroform, DMF, 0.5% Et_2NCS_2Na in DMF and DMF. A solution of the protected peptide MMP-substrate, Boc-Ala-Tyr(NO_2)-Gly-Pro-Leu-Gly-Leu-Tyr(Bu^t)-Ala-Arg(Pmc)-Lys(Abz (Boc))-Gly-OH (3 eq., 264 mg), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (2.88 eq., 40.5 µl) and *N*-ethylmorpholine (4 eq., 22.5 µl) in anhydrous DMF was added to the resin and allowed to react for a period of 14 h, where after the resin was washed with DMF, Fmoc was removed, and the resin washed with DMF and dichloromethane. The resin was dried by lyophilisation for 1.5 h and treated with a cocktail composed

of trifluoroacetic acid:dichloromethane:H_2O:MeSPh:$(CH_2SH)_2$:tri-isopropylsilane in 66.5:20:5:5:2.5:1 for 3 h to remove peptide side-chain protective groups. The resin was washed with AcOH, dichloromethane, methanol, water, 0.1 M NaOH (aq.), treated with 0.1 M NaOH (aq.) for a period of 40 min, and washed with water, methanol, dichloromethane, and lyophilised.

Screening of the library.

After lyophilisation the dried resin (approx. 400 mg corresponding to 165,000 beads/different compounds) was added purified recombinant rabbit catalytic domain MMP-12 (8 ml, 100 nM in buffer 50 mM tris pH=7.5, 150 mM NaCl, 10 mM $CaCl_2$, 50 µM $ZnSO_4$, 0.05% (v/v) Brij-35). Incubation was carried out for a period of 22.5 h at room temperature after which the resin was washed with water, 10 mM TFA, water, saturated $NaHCO_3$, water. An amount of water just enough to swell it was added to the resin and approx. 1/50 of resin was placed on a glass plate (8 x 8 cm). The other side of the glass plate had been furnished with a line made with a yellow, fluorescent highlighting pen and the line was covered with a piece of transparent tape. The resin was spread out in a row beside the yellow line and while looking in a fluorescence microscope the beads were sorted in such a way that only those appearing to be approx. as dark as the black support underneath were pushed (with a steel needle) to the side to lie on top of the yellow, fluorescent line. After sorting the batch of resin all dark beads were transferred to another glassplate and sorting was continued with a new batch. Approximately 1,000 dark beads were collected manually by inspection under a fluorescence microscope and from these 112 of the most persistently dark beads were selected and washed with MeCN:water 7:3. Each bead

was placed on a MALDI-TOF target and irradiated for 1 h with Hg lamp. Water (0.2 µl) and matrix solution (10 mg α-cyano-4-hydroxycinnamic acid in 1 ml MeCN:water 7:3, 0.2 µl) were added to each bead and they were allowed to dry out before the mass spectrum was acquired. The sequences of these active sequences were determined by the mass differences between the peaks from the ladder fragments (Fig. 5).

Example 5

Direct design of inhibitor sequences from active sequences identified in the PEGA bead inhibitor library.

Sixteen sequences of phosphinate based peptide derivatives of formula (I) were resolved from some of those beads in the PEGA bead inhibitory library that remained dark after incubation with MMP-12. The sequences were used for the direct design of the soluble putative MMP-inhibitors shown in Table 1 (04-01 through 04-16).

Table 1. Sequences of soluble phosphinate based peptide derivatives designed directly from sequences identified on isolated beads from the solid phase combinatorial library of phosphinic peptides that remained dark after incubation with MMP-12

Derivative	P4	P3	P2	P2'	P3'	P4'
04-03	Thr	Leu	Tyr	Leu	Asp	Gly
04-04	Val	Leu	Tyr	Thr	Leu	Ser
04-05	Ile	Met	Tyr	Val	Lys	Phe
04-07	Thr	Leu	Tyr	Arg	Ala	Ile
04-09	Thr	Leu	Arg	Leu	Phe	Phe
04-10	Ile	Leu	Arg	Met	Ala	Pro
04-11	Ser	Leu	Phe	Arg	Asp	Ile
04-12	Leu	Met	Phe	Tyr	Leu	Ser
04-13	Ile	Met	Tyr	Tyr	Met	Thr
04-14	Lys	Phe	Tyr	Leu	Tyr	Ala
04-15	Tyr	Ile	Tyr	Thr	Met	Pro
04-16	Ser	Met	Ala	Tyr	His	Gly
04-17	Ile	Met	Arg	Leu	Ser	Glu
04-02	Ile	Leu	Leu	Asn	Leu	Ile
04-06	Leu	Ile	Glu	Arg	Lys	Gly
04-08	Glu	Phe	Tyr	Lys	Tyr	Asn

In all cases P1-P1' consisted of the -GlyΨ{PO_2H-CH_2}Leu moiety. In all cases except 04-03, 04-16 and 04-06, which terminate at P4', Gly constituted P5'.

Example 6.

Design of inhibitor sequences from a consensus sequence.

Reliable sequences were resolved in 82 out of 112 dark beads after incubation of the PEGA bead inhibitory library with MMP-12. The frequencies of occurrence of the different amino acids in the different subsites were determined giving the distributions shown in Fig. 6. From this it is seen that, in subsite P4 the most predominantly observed amino acid was Leu/Ile/Hyp and Lys/Gln, in P3 it was Met and Leu/Ile/Hyp, in P2 it was Tyr, Arg and Phe, in P2' it was Tyr and Leu/Ile/Hyp, in P3' it was Ala, Tyr, Met and Leu/Ile/Hyp, and in P4' it was Pro and Leu/Ile/Hyp. Based on these statistical observations as well as supporting observations of frequently occurring combinations of two or more amino acids, it was decided to use the sequence H-Leu-Met-Tyr-GlyΨ{PO_2H-CH_2}Leu-Tyr-Ala-Pro-OH as a consensus sequence. It should be noted that Leu in P4 was chosen irrespectively of the fact that it might as well have been the isobaric Ile or Hyp. However, when Edman degradation was used for sequencing of the three amino acids positioned N-terminally to the -GlyΨ{PO_2H-CH_2}Leu- moiety of the phosphinate based pseudopeptides of five selected beads, it was observed that Leu and Ile, but not Hyp, were present in P4 and P3. Thus, Leu (or Ile) seemed a more likely choice for the P4 position in the consensus sequence.

By systematically exchanging single amino acids in the different subsites of the consensus sequence or by truncation a further 25 sequences were designed (Table 2), that could give information about the amino acid preference in the difference subsites. In all cases P1-P1' consisted of the -Gly

$\Psi\{PO_2H\text{-}CH_2\}$Leu- moiety and except for the truncated sequences (04-01, 04-19, 04-20, and 04-21) a Gly constituted P5'. In the case of Gln in the P4-position, the *N*-terminal was acetylated due to problems with partial formation of pyro-Glu in 02-04. Peptide 02-22 was prepared in order to study the mechanism of binding of phosphinate based peptide derivative inhibitors to MMPs (see Example 10).

Table 2. The consensus sequence obtained from the PEGA-bead library after incubation with MMP-12, and substitutions of this consensus sequence

	Derivative	P4	P3	P2	P2'	P3'	P4'
Consensus sequence	**02-01**	**Leu**	**Met**	**Tyr**	**Tyr**	**Ala**	**Pro**
Substitution							
P4	02-02	**Ile**	Met	Tyr	Tyr	Ala	Pro
P4	02-03	**Lys**	Met	Tyr	Tyr	Ala	Pro
P4	02-04	**PGlu/Gln**	Met	Tyr	Tyr	Ala	Pro
P4	02-21	**Ac-Gln**	Met	Tyr	Tyr	Ala	Pro
P3	02-05	Leu	**Leu**	Tyr	Tyr	Ala	Pro
P3	02-06	Leu	**Ile**	Tyr	Tyr	Ala	Pro
P3	02-07	Leu	**Pro**	Tyr	Tyr	Ala	Pro
P2	02-08	Leu	Met	**Arg**	Tyr	Ala	Pro
P2	02-09	Leu	Met	**Phe**	Tyr	Ala	Pro
P2'	02-10	Leu	Met	Tyr	**Leu**	Ala	Pro
P2'	02-11	Leu	Met	Tyr	**Ile**	Ala	Pro
P2'	02-12	Leu	Met	Tyr	**Hyp**	Ala	Pro
P3'	02-13	Leu	Met	Tyr	Tyr	**Leu**	Pro
P3'	02-14	Leu	Met	Tyr	Tyr	**Ile**	Pro
P3'	02-15	Leu	Met	Tyr	Tyr	**Hyp**	Pro
P3'	02-16	Leu	Met	Tyr	Tyr	**Met**	Pro

P3'	02-17	Leu	Met	Tyr	Tyr	**Tyr**	Pro
P3'	02-22	Leu	Met	Tyr	Tyr	**Trp**	Pro
P4'	02-18	Leu	Met	Tyr	Tyr	Ala	**Leu**
P4'	02-19	Leu	Met	Tyr	Tyr	Ala	**Ile**
P4'	02-20	Leu	Met	Tyr	Tyr	Ala	**Hyp**
Truncated	04-01	-	-	Tyr	Tyr	-	-
Truncated	04-19	-	-	Tyr	Tyr	Trp	-
Truncated	04-20	-	-	Tyr	Tyr	Met	-
Truncated	04-21	-	Met	Tyr	Tyr	-	-
P4-P4'	04-18	**Ile**	**Leu**	**Phe**	**Leu**	**Met**	**Ile**

In all cases P1-P1' consisted of the -GlyΨ\{PO_2H-CH_2\}Leu- moiety In all cases except 04-01, 04-19, 04-20, and 04-21, a Gly constituted P5'.

Example 7

Sequential screening of a solid phase combinatorial library of phosphinic peptides (see also the schematic representation of the experimental set-up in Fig. 7).

The dried resin (approx. 0.8 g, corresponding to 330,000 beads) was swelled in water, split in three equal sized portions of approx. 265 mg each and lyophilised. After lyophilisation, 2 ml of either MMP-9, MMP-13 or MMP-14 in 10 mM Tris, 0.1 M NaCl, 10 mM CaCl2, 50 mM ZnCl2, 0.05 w/v % Brij, pH 7.50 was added per 100 mg of beads. Incubations was carried out at ambient temperature, in the dark for 24 hours. A small volume of highly concentrated proteinase was added twice for MMP-9 during the 24 hrs incubation. The total proteinase concentrations were 370 nM

for MMP-9, 170 nM for MMP-13, and 115 nM for MMP-14. After incubation, the resin was washed using 2 % TFA, water, TFA, water, buffer, water and buffer. An amount of water enough to swell the resin was added and approx. 1/50 of the resin was placed on a glass plate (8.5 x 8 cm). The backside of the plate was furnished with a line made by a yellow flourescent highlightening pen covered by transparent tape. A small fraction of the beads were placed under the microscope and sorted by the use of a steel needle while looking in the fluorescence microscope. Dark beads were pushed to lie on top of the flourescent line. All the selected dark beads (apart from the beads obtained from the primary incubation with MMP-14) were inspected once more and persistently dark beads were split in two and transferred to plastic syringes equipped with a filter stopper. The subsequent secondary incubation was done in these syringes. Approximately 1,000 dark beads were selected from each of the primary incubations. The resin was drained of water and 200-400 µl 200 nM MMP-9, or 100 nM MMP-13 or MMP-14 was added to the resin. Incubations were carried out for one hour at ambient temperature, in the dark. After incubation, the beads were washed and sorted as described above, except that this time fluorescent beads were saved for sequence analysis by MALDI-TOF as described in Example 4. The dark beads were transferred into a new syringe with a filter stopper and incubated a third time by the same procedure as used for the secondary incubations. Beads that were fluorecent after the third incubation were also saved for sequence analysis by MALDI-TOF.

Example 8

Design of sequences from active beads identified after sequential incubations of the PEGA bead inhibitor library.

Phosphinate based peptide derivatives from all of the 209 beads belonging to one of the twelve groups of beads that were fluorescent after the second or third MMP incubation (see Fig. 7) were sequence analysed by MALDI-TOF. For each of ten of the twelve groups, four sequences were selected as shown in Table 3 (05-01 to 05-40). None of the 79 sequences belonging to the last two groups of beads were selected, since these beads though remaining dark after the primary incubation with MMP-14 and becoming fluorescent during the secondary incubations with MMP-9 (+14-9) or MMP-13 (+14-13) may have been false positives. Decisions to choose between Leu, Ile and Hyp or between Lys and Gln which have the same molecular mass and therefore cannot be distinguished by mass spectrometry analysis, included comparisons of the 131 sequences from the sequential incubations with the sequences shown in Table 1 and Table 2. Furthermore, Hyp was chosen if Pro and/or Hyp was present in that particular position in either several of the sequences from the sequential incubations or in compounds that showed K_i values of less than 1 μM against the enzyme of interest. If not, Leu was chosen. The choice between Lys and Gln was made on the basis on the appearance of Asn and Arg in the same position. In ambiguous cases, both the Lys and Gln form of the same sequence were synthetised.

Table 3. Sequences of soluble phosphinate based peptide derivatives designed directly from sequences identified on beads isolated from the solid phase combinatorial library of phosphinic peptides and representing different groups after sequential incubations with MMP-9, MMP-13 and MMP-14.

Group	Compound	P4	P3	P2	P2'	P3'	P4'
+13-9	05-01	Thr	Ala	Ser	Met	Phe	Gly
	05-02	Met	Tyr	Thr	Tyr	Lys	Leu
+13-14	05-03	Thr	Arg	Lys	Ser	Glu	Leu
	05-04	Thr	Arg	Gln	Ser	Glu	Leu
+13+14-9	05-05		Ser	Met	Leu	Tyr	Ala
	05-06	Leu	Ala	Ala	Tyr	Phe	Tyr
+13+9-14	05-07	Glu	Ser	Asn	Tyr	Tyr	Gly
	05-08	Hyp	Val	Ala	Ser	Thr	Gly
+14+9-13	05-09	Hyp	Tyr	Met	Leu	Gln	Leu
	05-10	Hyp	Tyr	Met	Leu	Lys	Leu
+14+13-9	05-11	Val	Phe	Lys	Met	Ala	Lys
	05-12	Asn	Arg	Ala	Phe	Gln	Ala
+9-13	05-13	Arg	Val	Ser	Asn	Tyr	Gly
	05-14	Gly	Hyp	Lys	Tyr	Ans	Arg
+9-14	05-15	Gly	Hyp	Phe	Glu	Ser	Leu
	05-16	Val	Ser	His	Ala	Thr	Phe
+9+13-14	05-17	Tyr	Pro	Glu	Ser	Ala	Ser
	05-18	Hyp	Met	Val	Leu	Gln	Phe
+9+14-13	05-19	His	Phe	Lys	Gln	Gly	Phe
	05-20	Gln	Pro	His	Phe	Tyr	Asp
+13-9	05-21	Ser	Hyp	Asp	Gly	Val	Glu
	05-22	Arg	Hyp	Asp	Thr	Leu	Hyp

+13-14	05-23	Arg	Pro	Pro	Leu	Leu	Gly
	05-24	Lys	Tyr	Phe	Gly	Pro	Met
+13+14-9	05-25	Gly	Met	Gly	Pro	Phe	Leu
	05-26	Thr	Asn	Pro	Asn	Val	Glu
+13+9-14	05-27	Gly	Thr	Val	Ala	Lys	Gln
	05-28		Hyp	Leu	Leu	Phe	Hyp
+9-13	05-29	Lys	Thr	Met	Val	Gln	Leu
	05-30	Lys	Thr	Met	Val	Gln	Leu
+9-14	05-31	Tyr	Met	Arg	His	Ser	Gly
	05-32	Asn	Val	Val	Tyr	Leu	Glu
+9+13-14	05-33	Asp	Ala	His	Asp	Phe	Gly
	05-34	Thr	Pro	Leu	Glu	Ala	Asp
+9+14-13	05-35	Ala	Pro	Ala	Leu	Ala	Gln
	05-36	Arg	Pro	Ala	Gln	Met	Arg
+14+9-13	05-37	Tyr	Ala	Tyr	Lys	Tyr	Glu
	05-38	Tyr	Ala	Tyr	Gln	Tyr	Glu
+14+13-9	05-39	Thr	Hyp	Glu	Val	Ala	Gly
	05-40	Val	Ala	Lys	Gln	Arg	Gly

In all cases P1-P1' consisted of the -Gly$\Psi\{PO_2H-CH_2\}$Leu- moiety.

The compounds that have Gly in position P4' terminates at this position. In all other compounds, Gly constituted P5'.

Example 9

Kinetic analyses of novel substrate-mimicking phosphinate-type MMP inhibitors and of a phosphinate-type MMP inhibitor synthesized on solid phase by a direct approach.

Analyses of substrate mimicking phosphinate based peptide derivatives

The 14 substrate-mimicking phosphinate based peptide derivatives 01-01A to 01-07B (see Table 4) were analysed kinetically by first pre-incubation in various concentrations with either MMP-1, MMP-3, MMP-9, MMP-12 or MMP-14 for 30 min at 37 °C and then incubation of the mixture with the quenched fluorogenic peptide substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (#M1895, Bachem, Switzerland), where Mca is (7-methoxycoumarin-4-yl)acetyl and Dpa is (2´,4´-dinitrophenyl)-L-2,3-diaminopropionyl. The rate of reaction was compared to the rate without inhibitor, and if possible the ratios were used for calculation of the K_i-values for each combination of inhibitor and MMP (Table 4).

For all of the 7 phosphinate based peptide derivatives, the diastereomeric A-form, which eluted first in HPLC purification, was more potent than the corresponding B-form, which eluted last in HPLC. 01-07A, which mimics the very sensitive synthetic MMP-substrate, #M1895, was the most potent inhibitor of MMP-1, MMP-9 and MMP-14, and together with 01-04A the most potent inhibitor of MMP-3 and MMP-12. A K_i-value of 1.2 nM was observed for 01-07A with MMP-9. Similar values were obtained for 01-07A with MMP-2 (2.2 nM) and MMP-13 (1.5 nM), whereas it showed increasing K_i-values with MMP-14 (10 nM), MMP-20 (30 nM), MMP-1 (60 nM), MMP-7 (75 nM), MMP-12 (200 nM) and MMP-3 (2,200 nM). Also, the affinity towards a member of the MMP related ADAM family, ADAM-17 or TACE (TNF converting enzyme) was measured, giving a K_i value of 7,000 nM. These data indicate

that compound 01-07A is a rather selective inhibitor of gelatinases and collagenases. A sub-micromolar K_i-value was observed for a few combinations of MMP and natural substrate-mimicking inhibitor (01-01 to 01-06): 01-01A with MMP-9 and MMP-12, 01-03A with MMP-9, and 01-04A with MMP-3 and MMP-12, but it was never below 250 nM.

Table 4. Kinetic analyses of substrate-analogue phosphinate based peptide derivatives

No.	Sequence	MMP-1	MMP-3	MMP-9	MMP-12	MMP-14
01-01A	VAYG^{PC}LKSRG	$8.0\ 10^{-6}$	$9.9\ 10^{-6}$	$2.9\ 10^{-7}$	$7.9\ 10^{-7}$	$3.8\ 10^{-6}$
01-01B		$8.3\ 10^{-5}$	$1.4\ 10^{-4}$	$7.8\ 10^{-6}$	$3.6\ 10^{-5}$	$6.1\ 10^{-5}$
01-02A	AYG^{PC}LKSG	$2.1\ 10^{-5}$	$5.7\ 10^{-5}$	$3.5\ 10^{-6}$	$8.6\ 10^{-6}$	$2.0\ 10^{-5}$
01-02B		$2.7\ 10^{-4}$	$3.7\ 10^{-4}$	$7.0\ 10^{-5}$	$2.0\ 10^{-3}$	n.i.
01-03A	VYG^{PC}LRSG	$4.5\ 10^{-6}$	$2.4\ 10^{-5}$	$6.8\ 10^{-7}$	$6.0\ 10^{-6}$	$6.3\ 10^{-6}$
01-03B		$6.7\ 10^{-5}$	$2.2\ 10^{-4}$	$1.6\ 10^{-5}$	$1.4\ 10^{-4}$	$2.5\ 10^{-5}$
01-04A	GLAG^{PC}LWLPG	$2.2\ 10^{-4}$	$5.7\ 10^{-7}$	$1.7\ 10^{-5}$	$4.4\ 10^{-7}$	$3.2\ 10^{-6}$
01-04B		$3.5\ 10^{-4}$	$1.9\ 10^{-5}$	$3.9\ 10^{-4}$	$1.6\ 10^{-5}$	$1.1\ 10^{-4}$
01-05A	LAG^{PC}LWLG	$2.1\ 10^{-5}$	$3.8\ 10^{-6}$	$1.1\ 10^{-5}$	$1.6\ 10^{-6}$	$1.7\ 10^{-5}$
01-05B		$1.0\ 10^{-3}$	$9.4\ 10^{-5}$	$1.5\ 10^{-4}$	$9.1\ 10^{-5}$	n.i.
01-06A	LAG^{PC}LQLG	$2.5\ 10^{-4}$	$7.7\ 10^{-6}$	$8.4\ 10^{-6}$	$8.5\ 10^{-6}$	$1.6\ 10^{-5}$
01-06B		$2.4\ 10^{-4}$	$3.1\ 10^{-4}$	$2.9\ 10^{-5}$	$9.8\ 10^{-4}$	n.i.
01-07A	AGPLG^{PC}LYARG	$6.0\ 10^{-8}$	$2.2\ 10^{-6}$	$1.2\ 10^{-9}$	$2.0\ 10^{-7}$	$1.0\ 10^{-8}$
01-07B		$2.8\ 10^{-7}$	$8.2\ 10^{-6}$	$3.4\ 10^{-9}$	$3.2\ 10^{-6}$	$6.2\ 10^{-7}$

n.i.: no measurable inhibition.

Analyses of a phosphinate based peptide derivative synthesized on solid phase by a direct approach

The mean+/-SD of the K_i values of 03-01A were determined to be 86+/-17, 40+/-10, 19+/-2 and 27+/-4 μM for MMP-9, MMP-12, MMP-13, and MMP-20, respectively, thus, indicating that phosphinate based peptide derivatives containing an -Alaψ{P(O)(OH)-CH_2}Gly-moiety may not be so efficient MMP-inhibitors.

Example 10

Kinetic analyses of phosphinate-type MMP inhibitors derived from a PEGA-bead library incubated with MMP-12

Sequences of phosphinate based peptide derivatives found on dark beads isolated after incubation of the X^1-X^2-X^3-Glyψ(PO_2-CH_2)Leu-X^4-X^5-X^6-designed PEGA-bead inhibitor library with MMP-12, were synthesized (04-02 to 04-17, see Table 1) and tested for inhibitory activity towards MMPs. Also the consensus sequence LMYGψ(PO_2CH_2)LYAPG (02-01) and selective amino acid substitutions thereof (02-02 to 02-22, see Table 2), as well as truncated forms of the consensus sequence (04-01 and 04-21) and of 02-22 (04-19) and of 02-16 (04-20), were synthesized and tested.

In preliminary kinetic analyses, MMP-12 and MMP-9 were preincubated with various concentrations of each of the two diastereomeric forms of the phosphinate based peptide derivatives in order to determine the approximate IC_{50}-values. As was the case for all of the 7 substrate-mimicking inhibitors, 01-01 to 01-07, all of the PEGA-bead derived inhibitors, except for the almost inactive 02-12, were more potent in the diastereomeric A-form (which elutes first in HPLC purification) than in the corresponding B-form (which elutes last in HPLC). Thus, all further analyses were made with just the A-forms of the phosphinate based peptide derivatives of series 02 and 04.

Various MMPs (1-15 nM) were preincubated for 60 min at 37 °C with 8 or 14 different concentrations of each phosphinate based peptide derivative. Furthermore, each MMP was incubated alone in order to determine the rate of reaction of the uninhibited proteinase. The concentrations of the phosphinate based peptide derivatives were from 0.1 to 10 times the K_i-value, determined from preliminary experiments. The incubation buffer used for all MMPs, except MMP-3 contained 10 mM Trizma Base, 10 mM $CaCl_2$, 50 µM $ZnCl_2$, 0.1 M NaCl with the pH adjusted to 7.50 by addition of HCl. A 10 mM MES, 5 mM $CaCl_2$, 100 µM $ZnCl_2$, pH 5.0 buffer was used for MMP-3 measurements. In experiments with ADAM-17/TACE a 20 mM Trizma Base, 10 mM $CaCl_2$, 50 µM $ZnCl_2$, pH 8.0 buffer was used. The preincubations were carried out with 0.05 % w/v Brij 35 added to the buffer. The rate of reaction was measured using the quenched fluorogenic peptide substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (#M1895) from Bachem, Switzerland, where Mca is (7-methoxycoumarin-4-yl)acetyl and Dpa is (2´,4´-dinitrophenyl)-L-2,3-diamino-propionyl in a final concentration of 5 µM (MMP-7 or MMP-12), 3 µM (MMP-1 or MMP-14), 2 µM (MMP-13) or 1 µM (MMP-2 or MMP-9). For MMP-3, a final concentration of 2 µM Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 (#M2110 from Bachem) was used, while 0.5 µM Mca-Pro-Leu-Ala-Gln-Ala-Val-Dap(Dnp)-Arg-Ser-Ser-Ser-Arg-NH_2 (#M2255 from Bachem) was used for assaying ADAM-17/TACE. The reaction was initiated by mixing the preincubated enzyme-inhibitor solution 1:1 with the prediluted substrate solution.

The K_i-value for each pair of MMP and phosphinate based peptide derivative was calculated from plots of the rate of reaction obtained in presence of inhibitor relative to the rate of reaction in absence of inhibitor:

$$I_{Rel,[I]} = (I_{[I]\infty} \times [I]) / (K_i + [I])$$

where [I] is the concentration of the inhibitor, and $I_{Rel,[I]}$ and $I_{[I]\infty}$ are the relative inhibition at [I] and the asymptotically

measured relative inhibition at indefinitely high concentration of I, respectively.

Of the sixteen phosphinate based peptides designed directly from sequences found in the library after incubation with MMP-12, five (04-09A, 04-10A, 04-13A, 04-15A, and 04-17A) showed strong inhibition of MMP-12 with K_i values in the low nanomolar range. Another eight compounds showed intermediate inhibiton of MMP-12 with K_i values of less than 1 μM, while the apparent inhibitory activity observed while on solid phase for three compounds (04-02A, 04-06A, and 04-08A) could not be confirmed when they were tested in soluble form. A few of the compounds showed selective inhibition of MMP-12, with 04-15A being the most prominent example (40 to 2,000 times higher K_i values against MMP-7, -9, -13, and -14).

Table 5. Kinetic analyses of soluble phosphinate based peptide derivatives designed directly from sequences identified on beads isolated from the solid phase combinatorial library after incubation with MMP-12

Phosphinic Peptide	K_i MMP-12 nM	K_i MMP-9 nM	K_i MMP-13 nM	K_i MMP-14 NM	K_i MMP-7 nM
04-03A	66±2	49,000±10,000	800±100	130±4	400±140
04-04A	985±315	270,000±50,000	39,000±3,000	4,800±500	1,200±500
04-05A	74±9	31,000±5,000	4,900±800	116±6	75±24
04-07A	875±465	32,000±5,000	2,200±200	1,350±10	4,600±300
04-09A	22±5	1,130±170	335±90	24±2	260±95
04-10A	25±4	56±4	20±10	20±5	455±70
04-11A	762±8	180,000±12000	1,500±1,400	1,500±300	4,700±400
04-12A	129±5	24,000±6,000	5,100±2,000	190±100	42±2
04-13A	8±4	7,600±1,200	1,500±100	450±120	100±35
04-14A	150±18	4,400±300	4,200±500	514±5	265±65
04-15A	19±15	35,000±13,000	3,000±1,000	4,500±1,700	750±90
04-16A	303±9	6,000±400	2,700±1,000	10,800±1,000	175±25
04-17A	23±8	400±80	204±51	190±33	1,700±200

04-02A	23,000±1,000	≥ 100,000	311,000±56,000	≥ 75,000	29,000±11,000
04-06A	≥389,000±38,000	16,800±800	32,000±5,000	2,100±200	2.300±1,700
04-08A	3,700±500	300,000±50,000	128,000±10,000	77,000±15,000	32,000±12,000

With a few exceptions, all of the soluble phosphinate based peptide derivatives (02-01A to 02-22A, 04-01A, 04-19A to 04-21A, and 04-18A), which were synthesized according to their relationship to the consensus sequence (02-01), showed strong inhibition of MMP-12, with Ki-values in the low nanomolar range (Table 6). The exceptions were the truncated forms (04-01A and 04-19A to 04-21A), which were 150 to 20,000 times weaker than their corresponding full length forms, and the 2,500 fold reduction in potency, which was observed when the Tyrosine in the P2´ position of the consensus sequence was substituted by a Hydroxyproline.

The Ki-values were also determined for each of the derivatives by incubation with MMP-9, MMP-13, MMP-14, MMP-7, and MMP-20 (Table 6). The selectivity of each inhibitor towards MMP-12 was calculated as the ratio between the Ki-value for MMP-12 and the Ki-value for any other MMP. From these selectivity numbers, it is particularly striking that the P3'-site provides a possibility of increasing the selectivity towards MMP-12 versus MMP-9 and MMP-13, when the Alanine of 02-01A is substituted by a Leucine (02-13A), Isoleucine (02-14A), Methionine (02-16A), Tyrosine (02-17A) or Tryptophan (02-22A). Furthermore, the K_i-values of 02-07A show that MMP-9 and MMP-13 favours a backbone bend introduced by substituting Methionine in 02-01A with Proline in the P3-site, whereas this substitution does not increase the inhibition of MMP-12. In inhibitors 02-12A and 02-15A a Hydroxyproline was introduced in positions P2', and P3', respectively. Inhibitor 02-12A shows that neither MMP-12

nor MMP-9 or MMP-13 can accommodate a backbone bend in P2', whereas the 02-15A inhibitor shows that inhibition of MMP-12 is only moderately reduced when replacing the Alanine in P3' of 02-01A with Hydroxyproline. The affinity of MMP-9 and MMP-13 for 02-15A is more severely impeded by this change, which also reflects that small amino acids like Alanine are favoured very much by MMP-9 and MMP-13 in this position. For this position, it is also interesting to notice that MMP-14 is inhibited equally when this Alanine is substituted by a larger amino acid like Leucine. In fact the substitution to Methionine (02-16A) increases the affinity towards MMP-14.

A "second generation" consensus sequence compound (04-18A) was synthesized according to the results of the kinetic analyses of 02-01A to 02-22A. Compound 04-18A contained those amino acids, which for each subsite gave the most potent inhibitor in the previous single amino acid substitutions of the consensus sequence 02-01A. However, when compared to the original consensus sequence, 04-18A was not a more powerful inhibitor of MMP-12 and only marginally better towards MMP-14 (and MMP-7), whereas it was 40 to 100 times less efficient towards MMP-9 and MMP-13. Probably, these changes were mainly due to the substitution of Alanine in P3' with Methionine, as discussed above, and the pattern of inhibition by 04-18A resembled 02-16A very much. Thus, as has been suggested previously (Nagase *et al*, 1994) for petide-like substrates of MMPs, the additivity principle is not easily applicable for single position amino acid substitutions.

Table 6. Kinetic analyses of soluble phosphinate based peptide derivatives related to the consensus sequence (02-01) obtained from the PEGA-bead library after incubation with MMP-12

Phosphinic peptide	K_i MMP-12 NM	K_i MMP-9 nM	K_i MMP-13 NM	K_i MMP-14 nM	K_i MMP-7 nM	K_i MMP-20 nM
02-01A	16±4	133±8	34±6	156±29	77±7	50±7
02-02A	13±2	120±20	30±2	90±10	43±11	203±9
02-03A	17±5	143±18	77±30	155±52	200±20	43±8
02-04A	27±7	44±6	38±2	222±5	50±1	227±10
02-21A	23±5	65±7	35±1	260±120	32±8	32±8
02-05A	13±5	110±10	17±2	148±31	82±4	46±6
02-06A	22±4	19±1	5±1	630±215	117±8	70±7
02-07A	28±7	4±1	1.8±0.3	232±32	449±7	32±2
02-08A	20±6	20±5	65±7	112±7	330±130	78±9
02-09A	19±3	150±45	74±7	161±38	46±5	22±1
02-10A	9±2	200±50	96±3	25±5	28±2	9.4±0.4
02-11A	12±2	200±50	236±15	80±30	29±2	9.7±0.8
02-12A	41,000±18,000	39,000±7,000	13,900±900	187,000±3,000	137,000±27,000	4,900±400
02-13A	8±2	9,000±2,000	2,300±200	195±65	9.4±0.9	705±57

02-14A	16±3	7,100±600	1,800±200	855±275	59±5	260±10
02-15A	85±14	12,000±1,000	5,400±400	3,200±700	735±45	1,610±30
02-16A	6±1	2,900±600	700±200	36±6	21±4	220±9
02-17A	6±1	1,200±500	400±40	92±7	17±1	130±10
02-22A	21±6	2,600±600	700±15	270+±210	79±16	222±45
02-18A	30±8	710±60	108±34	203±23	129±8	196±21
02-19A	20±4	715±265	71±6	185±11	76±10	143±20
02-20A	15±5	120±20	40±12	193±3	95±35	55±5
04-01A	350,000±90,000	340,000±90,000	395,000±135,000	nd	nd	nd
04-19A	58,000±7,000	nd	487,000±81,000	287,000±22,000	nd	nd
04-20A	14,600±2,100	nd	141,000±20,000	90,000±4,000	112,000±33,000	nd
04-21A	2,500±500	nd	8,200±1,500	nd	nd	nd
04-18A	28±2	5,100±200	4,600±2,900	27±3	31±10	nd

To investigate the binding mechanism of phosphinate based peptide derivatives to MMP-12, 02-22A was applied as a model inhibitor, since it contains a Tryptophan in P3'. The Tryptophan residue provides a fluorescent probe which is known to change its fluorescence upon burial in a protein moiety. The kinetics of the binding can thus be measured directly as change of fluorescence in the inhibitor-enzyme solution, as opposed to the other inhibitors where binding is measured indirectly by the activity towards a substrate. Furthermore, 02-22A has 120 times higher affinity towards MMP-12 than MMP-9, which allows the study of the binding mechanism of both a strong and a relatively weak inhibitor. Here only data for the binding of 02-22A to MMP-12 are shown. The incubations were performed in 10 mM TrizmaBase, 0.1 M NaCl, 10 mM $CaCl_2$, 50 µM $ZnCl_2$, pH 7.5 at 37°C.

The binding was followed by monitoring the change of fluorescence in a stopped-flow instrument (2 msec dead time) using 280 nm excitation and a 335 nm cut-off filter in the detector. The pseudo-first order rate of binding showed saturation at high concentrations of inhibitor, and the amplitude remained constant over the concentration range examined. This suggest a two-step binding mechanism:

$$E + I \underset{}{\overset{K_1}{\rightleftarrows}} E - I \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI^*$$

where E is enzyme, I is inhibitor, E-I is an intermediate complex rapidly formed with relatively low affinity and EI* is the final complex, which is the one giving the change in fluorescence.

The value of the first dissociation constant K_1 is 93±13 µM, and k_2 is 2.6±0.2 s^{-1}. The value of k_{-2} $(5.9\pm0.7)\times10^{-4}$ s^{-1} was too small to be calculated directly, and was thus calculated from the overall K_i-value ($K_i = K_1 \times K_2$; $K_2 = k_{-2}/k_2$) shown in Table 3 (21 ± 6 nM). These data suggest that the slow conversion from

E-I to EI* involves a conformational change of either the inhibitor or the enzyme or both.

Example 11.

Kinetic analyses of phosphinate-type MMP inhibitors derived from a PEGA-bead library sequentially incubated with MMP-9, MMP-13, and MMP-14.

Forty phosphinate based peptide derivatives, with sequences selected among 131 sequences identified by sequential incubations of the PEGA-bead libray with MMP-9, MMP-13 and MMP-14, were analysed (see Table 7) in soluble form for inhibitory activity against these three MMPs as described in Example 10. Apparently, compound 05-34 belonging to the group (+9+13-14) was the only phosphinic peptide that gave the anticipated pattern of inhibiton by showing moderate K_i values of 560 nM and 100 nM for MMP-9 and MMP-13, respectively, and an approx. 100 times higher K_i value for MMP-14). Furthermore, compounds 05-09, 05-10, 05-20 and 05-35 showed inhibition patterns that lived up to some, but not all of the expectations. Compounds 05-20 and 05-35 (both from the +9+14-13 group) showed relatively strong inhibition of all three MMPs, while 05-09 and 05-10 (both from the +14+9-13 group) inhibited MMP-14 quite selectively. Thus, according to the selection procedure 05-09, 05-10, 05-20 and 05-35 should all be able to inhibit MMP-14 and MMP-9 but not MMP-13. Kinetic analyses of PEGA-bead library phosphinic peptides from sequential incubations.

It is worth noting that the Series 05 phosphinic peptides were tested as unpurified products, i.e. containing both the A and B diastereomeric form in a mixture as well as small amounts of impurities and NaCl. Further purification may lead

to more convincing results, and at least will reduce the K_i-values, since the concentrations of the MMP-inhibitory A-form constitute just half (or slightly less) than the total amounts of the A/B-mixtures, which were used for the calculations in Table 7.

Table 7. Kinetic analyses of soluble phosphinate based peptide derivatives designed directly from sequences identified on beads isolated from the solid phase combinatorial library after sequential incubations with MMP-9, MMP-13, and MMP-14.

Group	Phosphinic peptide	K_I MMP-9 µM	K_I MMP-13 µM	K_I MMP-14 µM
+13-9	05-01AB	6±1	6.9±0.8	8±1
	05-02AB	22±2	48±4	6.1±0.9
+13-14	05-03AB	≥250	58±5	260±45
	05-04AB	500±117	340±26	87±7
+13+14-9	05-05AB	24±5	21±1	1.4±0.3
	05-06AB	0.080±0.010	0.11±0.04	1.7±0.1
+13+9-14	05-07AB	105±18	73±4	0.56±0.05
	05-08AB	5.1±0.8	3.3±0.1	4±1
+14+9-13	05-09AB	17±3	27±2	0.042±0.009
	05-10AB	14±5	42±21	0.210±0.010
+14+13-9	05-11AB	6±1	6.5±0.7	0.8±0.3
	05-12AB	12±5	22±4	1.6±0.3
+9-13	05-13AB	19±5	22±2	14±6
	05-14AB	534±112	960±100	140±27
+9-14	05-15AB	≥480	106±15	166±56
	05-16AB	113±26	39±2	9±1
+9+13-14	05-17AB	1.7±0.6	0.850±0.028	1.0±0.3
	05-18AB	≥40 NS!	39±4	1.1±0.5

+9+14-13	05-19AB	15±3	22±3	6±1
	05-20AB	0.19±0.03	0.13±0.03	0.60±0.15
+13-9	05-21AB	33±8	2.1±0.9	≥100
	05-22AB	26±4	1.4±0.4	≥25
+13-14	05-23AB	26±8	32±8	50±8
	05-24AB	270±40	100±65	≥50
+13+14-9	05-25AB	36±18	0.65±0.24	≥25
	05-26AB	27±10	0.8±0.4	≥25
+13+9-14	05-27AB	117±95	7±9	≥25
	05-28AB	33±14	2.3±0.6	≥25
+9-13	05-29AB	228±54	30±39	8±3
	05-30AB	178±15	29±8	≥20
+9-14	05-31AB	3.6±0.6	11±4	12±3
	05-32AB	nd	nd	Nd
+9+13-14	05-33AB	66±6	≥25	≥25
	05-34AB	0.56±0.08	0.100±0.02	32±2
+9+14-13	05-35AB	0.039±0.008	0.011±0.003	0.13±0.01
	05-36AB	6±2	4.6±0.5	8±2
+14+9-13	05-37AB	2	0.8 ±0.3	18±9
	05-38AB	2.5	1.0±0.3	12±2
+14+13-9	05-39AB	≥25	2.8±0.8	≥250
	05-40AB	423±95	235±70	≥250

Example 12. *The collagen invasion assay.*

Inhibition of osteoclast invasion through type I collagen.

In order to study the role of osteoclast-derived MMPs in the recruitment and invasion of osteoclasts, we have developed a method which combines osteoclast purification and characterisation of their proteinase-dependent invasion through type I collagen. Briefly, purified or non-purified

osteoclasts were seeded on the membrane of tissue culture inserts (Costar, 12 μm pore size) which was coated with a type I collagen gel (Nitta Collagen, Japan; 10 $\mu l/cm^2$ of 3 mg/ml type I collagen pre-incubated at 37°C). Osteoclast migration to the lower surface of the membrane was studied by quantitative microscopy after an overnight culture in the absence or presence of MMP inhibitors. We found that both purified and non-purified osteoclast could extend cell processes into the pores of the membranes and spread over the lower membrane surface. We have previously shown that this migration process is inhibited by the general hydroxamate-type MMP inhibitors, RP59794 and BB-94, and by the natural MMP inhibitor, TIMP-2, but not by inhibitors of non-MMP proteinases (Sato *et al*, 1998). This indicates that osteoclasts themselves can overcome a collagen barrier by migrating through it without the participation of other cells and via a metalloproteinase dependent pathway.

We have applied the novel phosphinate based peptide derivatives, 01-02B, 01-01A, 01-07A and 01-07B, in this model and found a strict correlation between the K_i of the novel inhibitors towards MMP-9, and their ability to inhibit osteoclast invasion through type I collagen gel (Fig. 8 and Table 4 of Example 9). When used in a concentration of 10 μM, the most potent of the phosphinate-type inhibitors, 01-07A, (K_i = 0.6 nM towards MMP-9), reduced invasion with approx. 80%, which corresponds well to the potency of the previously studied hydroxamate-type MMP inhibitors.

Thus, the phosphinate-type MMP inhibitors are at least as potent as the previously known general MMP inhibitors in reducing osteoclast invasion through collagen.

Example 13. *The pericellular collagenolysis assay*

Inhibition of osteoclastic pericellular collagenolysis.

In order to study the osteoclastic interaction with and movement over a surface of type I collagen, rabbit or murine bone cells were seeded and cultured on glass coverslips coated with type I collagen. The cells were cultured in the absence or presence of the hydroxamate-type MMP inhibitor GM6001. After overnight culture, the collagen coating was studied by immunolocalisation with antibodies against type I collagen. Collagen-free zones surrounding the osteoclasts were evidence for a pericellular collagenolytic activity of the osteoclast. The areas of the collagen-free zones were significantly reduced (approx. 80%) in the presence of GM6001 (Fig. 9). To investigate whether the effect of MMP inhibitors is also reflected in cell motility, time-lapse camera assisted studies of osteoclasts seeded on a two-dimensional type I collagen coating were performed. The migratory distance of the osteoclasts was significantly reduced in the presence of GM6001. Furthermore, there was a strict correlation between the migrated path of the osteoclast and the collagen free zones (Fig. 10).

Example 14. *The bone lining cell invasion assay*

Inhibition of osteoclast invasion through bone lining cells

In order to study the invasion of osteoclasts through a layer of bone lining cells, MC3T3-E1 cells (a murine calvarial osteoblast-like cell line) were seeded at a density of $3x10^4$

per slice of bovine cortical bone (diameter 5.8 mm, thickness 150 μm in a 0.32 cm^2 plastic well of a 96 well tissue culture plate) and cultured for 24 hrs. This resulted in a bone surface covered by a confluent layer of osteoblasts mimicking the natural bone lining cells. For control purposes similar bone slices were prepared without seeding of cells.

Mixed bone cells constituting osteoclasts or purified osteoclast were subsequently seeded onto the bone slices in the absence or presence of a proteinase inhibitor. Bone resorption was quantified by staining for resorption pits and determination by computer-assisted microscopy of the resorbed surface area. Bone slices which were covered by an established layer of MC3T3-E1 cells had 40% less resorption than slices which were not covered by lining cells (Fig. 11). When bone was covered by lining cells, the MMP inhibitor, GM6001, reduced bone resorption with approx. 50% (Fig. 11), whereas it had no effect when the bone slices were not covered (data not shown here, but described by us in Sato *et al*, 1998). Thus metalloproteinases play an important role in osteoclast penetration of an established protecting bone lining cell layer.

Example 15. *The bone lining cell elongation assay*

Inhibition of TGF-β induced changes in bone lining cell morphology

In order to mimic the bone lining cell layer, osteoblasts were cultured until confluence on either plastic surface or on a bone substratum in tissue culture plates.

In brief, MC3T3-E1 cells were seeded in serum-free α-MEM on plastic in a density of $3x10^4$ per cm^2. After reaching confluence, the osteoblasts were cultured for 24 hours in the presence of 2.5 ng/ml TGF-β and in the absence or presence of 10 μM proteinase inhibitor. The cell-free area was quantified by computer-assisted microscopy. Varying from experiment to experiment 2.5 ng/ml TGF-β induced a 2-3 fold increase in cell-free area compared to untreated controls. The induced osteoblast elongation could be reduced by more than 50% by 10 μM of the hydroxamate-type MMP inhibitor, GM6001 but not by 10 μM of inhibitors of serine proteinases (aprotinin), cysteine proteinases (E-64), and aspartic proteinases (pepstatin) (Fig. 12). To clarify if the observed reduction in TGF-β induced osteoblast elongation was a general feature of MMP inhibitors or restricted to hydroxamates, the novel phosphinate-type MMP inhibitor 01-07A was compared to GM6001. At 10 μM, the two inhibitors were equally efficient (Fig. 13). Thus, metalloproteinases are important for the induction of morphological change in bone lining cells by TGF-β.

The relevance of TGF-β induced osteoblast elongation for bone resorption, and the effect of MMP inhibitors on this process, was studied by seeding $4.5x10^4$ MC3T3-E1 cells on slices of bovine cortical bone (diameter 5.8 mm, thickness 150 μm). The lining cells were cultured for 24 hrs in the absence or presence of 2.5 ng/ml of TGF-β and in the absence or presence of proteinase inhibitors. Bone slices without lining cells were prepared for control purposes. The bone slices were then treated with fixative (95% EtOH) for 20 min and washed carefully with culture medium. Subsequently, osteoclasts isolated from long bones of 10-day-old rabbits were seeded on

the bone slices and cultured for 72 hrs. Bone resorption was quantified by staining for resorption pits and determination of the resorbed surface area by computer-assisted microscopy. The presence of bone lining cells reduced pit formation by 50% compared to bone slice which were not covered by cells (Fig. 14). Stimulation of the bone cell layer with 2.5 ng/ml TGF-β almost recovered the bone resorption to the level observed for slices without cells. Stimulation of the lining cells with TGF-β in the presence of 10 μM GM6001 blocked the bone resorption to a level comparable to the one observed for slices with untreated lining cells (Fig. 14). Thus, bone resorption induced by TGF-β can be controlled specifically by MMP inhibitors through reduction of the elongation of bone lining cells.

The hypothesis that TGF-β induced cells shape changes in bone lining cells will lead to stimulated osteoclast resorption, was further tested in mouse calvariae cultures. In brief, ^{45}Ca-labelled calvariae from 18-day-old mouse foetuses were cultured for 6 days in the presence of 2.5 ng/ml TGF-β, which significantly stimulated decalcification compared to non-treated controls (Fig. 15). In order to investigate whether MMP-inhibition would reduce this stimulation of bone resorption in tissue culture, the novel phosphinate-type MMP inhibitor, 01-07A (10 μM) was added simultaneously with TGF-β. Under this condition, decalcification was markedly reduced by 01-07A (Fig. 16).

Example 16. *The metatarsal and tibia assays*

Inhibition of osteoclast migration and resorptive activity in metatarsal and tibia cultures

Pregnant NRMI mice were injected with 100 µCi ^{45}Ca s.c. at day 16 of gestation and sacrificed 24 hrs later. The three middle metatarsals (i.e., the "triad") and tibiae were isolated from the hind limbs of the 17-day-old foetuses in PBS, pH 7.4.

In tissue culture, the right triad or tibia from individual foetuses was used for treatment with MMP inhibitor and the corresponding left triad or tibia from the same animal was used as paired vehicle treated control. One triad and one tibia from each litter was killed at the start of the experiments by three freeze-thawing cycles and used for measuring the level of passive physico-chemical release of matrix bound radiolabelled calcium. Isolated triads and tibias were placed on pieces of filter paper (Millipore, 10 µm pore size) and allowed to preincubate for 2 hrs in 0.4 ml BGJb medium, supplemented with $NaHCO_3$ (2,200 mg/l), NaCl (900 mg/l), ascorbic acid (50 µg/l), and albumax (1 g/l), before the medium was replenished with medium containing MMP inhibitor or vehicle. The bones were cultured for up to seven days with conditioned media being replaced and collected at day 1, 2, 4 and 7 for analysis of ^{45}Ca release. At the last day of cultivation, triads and tibiae were demineralised in 5% acidic acid to recover the remaining ^{45}Ca.

The result is expressed as the ratio between the ^{45}Ca actively released in treated (T) bone (in %) and the corresponding ^{45}Ca actively released in the paired vehicle

treated control (C) bone (in %) during the same period, using equation I listed below:

$$^{45}Ca \text{ Release } (T/C) = \frac{(\%^{45}Ca_T - \%^{45}Ca_K)}{(\%^{45}Ca_C - \%^{45}Ca_K)} \quad (I)$$

The individual mean $\%^{45}Ca$ value from treated (T), untreated control (C) and killed control (K) bones is calculated as the ratio (in %) between the release of ^{45}Ca into the conditioned medium (in cpm) during the particular culture period and the total ^{45}Ca available in the individual bone (in cpm) at the initiation of the particular period, using equation II listed below:

$$\%^{45}Ca = (^{45}Ca \text{ (cpm)} / {}^{45}Ca \text{ (cpm)}_{total}) \times 100\% \quad (II)$$

For histological inspections, the triads were cultured for two or four days and then rinsed twice in PBS before fixation in 4% neutral-buffered formaldehyde for 3 hrs at 4°C, decalcification in 5% formic acid and 5% formaldehyde for 3 hrs at 4°C. Triads were then stained for TRAP by pararosanilin as coupler and 10 mM L(+)-tartratic acid as inhibitor, dehydrated in a graded series of ethanol and embedded in paraffin. Serial 5 µm sections were obtained by a microtome, counterstained with Ehrlich's haematoxylin for 30 seconds and finally mounted before quantification.

The hydroxamate-type MMP inhibitor, BB-94, dose-dependently reduced ^{45}Ca release in triads of metatarsals as compared to paired vehicle-treated triads (Fig. 17). This was seen both when triads were cultured under non-stimulated and

PTH-stimulated conditions where the mean ± SEM half inhibitory concentration (IC_{50}) for BB-94 was calculated to be 11 ± 3 nM (n = 4) and 23 ± 5 nM (n = 4), respectively, with Hill slopes of -1.1 ± 0.3 and -0.8 ± 0.2, respectively. As demonstrated by sections of triads stained for TRAP+ osteoclasts, BB-94 inhibited migration of maturing osteoclasts from the periosteal layer into the calcified matrix when administered at a concentration (3 μM) that completely blocks ^{45}Ca release (Table 8).

Table 8. Histomorphometric analysis on paired triads treated with vehicle or BB-94.

	Day 2			Day 4		
	OCs out	OCs in	%^{45}Ca	OCs out	OCs in	%^{45}Ca
Vehicle	8.8±0.9	10±1.3	18±1.7	9.2±1.2	14±0.5	47±4.2
BB-94	10±1.3	0.9±0.4$^{\#}$	3.5±1.2**	14±1.0*	0$^{\#}$	5.0±1.1**

Triads were cultured with vehicle as control or 3 μM BB-94 for 2 or 4 days when bones were fixed, decalcified and stained for TRAP-positive osteoclasts (OCs). The mean ± SEM number of osteoclasts outside (OCs out) or inside (OCs in) the calcified matrix was counted in three non-adjacent sections from each metatarsal (n = 16 from 4 independent experiments). The bone resorption is expressed as %^{45}Ca, which is the mean ± SEM calcium release in percentage of total calcium available corrected for killed control. Significance levels * $p < 0.05$, ** $p < 0.005$, # $p < 0.00001$.

In the tibia assay, BB-94 dose-dependently, but less potently than in the triads, reduced the level of ^{45}Ca release during basal and PTH-stimulated cultivation with calculated

IC_{50} values of 280 ± 71 nM and 55 ± 26 nM , (n = 4, for each condition) (Fig. 17). Furthermore, the inhibition of ^{45}Ca release in the tibiae was incomplete reaching 68 ± 7% and 59 ± 4% at 3 μM BB-94 under non-stimulated and PTH-stimulated conditions, respectively.

Similar results were observed for another hydroxamate-type MMP inhibitor, GM6001, which dose-dependently reduced the ^{45}Ca release from both metatarsal and tibia cultures with IC_{50} values of 22 ± 6 nM and 75 ± 8 nM, respectively and with Hill slopes of 0.93 ± 0.20 and 1.13 ± 0.10, respectively (Fig. 18). As seen for BB-94 the migration of osteoclasts from the periosteal layer into the calcified cartilage in metatarsals was totally abolished in the presence of 3 μM GM6001 (data not shown). In contrast to its complete inhibition in metatarsals, the inhibition in tibiae by GM6001 (like for BB-94) was not complete reaching a maximal 75% at micromolar concentrations (Fig. 18).

Also some of the novel phosphinate based peptide derivatives were efficient, dose-dependent inhibitors of decalcification in the metatarsal model (Table 9A). Among these, the most potent inhibitor of MMP-9, 01-07A (K_i = 1.2 nM), was able to inhibit the ^{45}Ca release in metatarsals with an IC_{50} value of 200 nM. As for BB-94 (K_i = 0.3 nM for MMP-9, IC_{50} in metatarsals approx. 15 nM) and GM6001 (K_i = 1.0 nM for MMP-9, IC_{50} in metatarsals approx. 20 nM), the migration of osteoclasts from the periosteal layer into the calcified cartilage according to histological inspection of the metatarsals was completely blocked in the presence of 2 μM 01-07A, thus strongly indicating that both hydroxamate-type and phosphinate based MMP-inhibitors can block the migration of osteoclasts. In order to increase the understanding of the

importance of individual MMPs in the decalcification of metatarsals, the correlations between the individual IC_{50} value of phosphinate based MMP-inhibitors in these tissue cultures and their K_i values towards various MMPs were investigated (Fig 19). This correlation study is a simplification of the probable complex interactions between various MMPs, but still it is interesting to notice that the K_i values of the inhibitors towards MMP-9 and MMP-13 are good predictors of their ability to reduce decalcification in the metatarsal cultures, whereas the seems to be no or even inverse proportionality for K_i values towards MMP-7, MMP-12, MMP-14, and MMP-20.

When applied to tibia cultures at 10 µM or even higher concentrations, none of the hitherto tested phosphinate-type inhibitors affected ^{45}Ca release (Table 9B), whereas the IC_{50} values of BB-94 and GM6001 in this assay were approx. 55 nM and 75 nM, respectively. This indicates, that the hydroxamate-type inhibitors also directly affect the removal of mineralised osseous substance by osteoclasts, whereas the phosphinate based peptide derivatives do not.

The two potent, hydroxamate-type MMP inhibitors BB-94 and GM6001, which have little selectivity (low or sub-nanomolar K_i-values) for a variety of MMPs including MMP-1, -2, -3, -7, -9, -12, -13, -14, and -20, inhibited the migration and reduced the resorptive activity of osteoclasts in bone cultures, whereas the potent MMP-9 selective phosphinate-type inhibitor, 01-07A (see Table 4 of Example 9), selectively inhibited the migration but did not affect the resorptive activity of osteoclasts.

Table 9 Effect of phosphinate based peptide derivatives on decalcification in bone tissue cultures

A. Metatarsals Cultures - Migration and resorption

Compound	IC50 in μM
GM6001	0.02 +/- 0.006
01-07A	0.20
01-07B	23
02-05A	10
02-06A	5
02-07A	0.9 +/- 0.2
02-08A	5
02-10A	14 +/- 8
02-11A	15
02-16A	23 +/- 6
04-18A	10 +/- 1
04-09A	7
04-10A	4 +/- 3
05-06A/B	7
05-10A/B	14 +/- 1
05-20A/B	11 +/- 2
05-23A/B	No Inhibition
05-24A/B	No Inhibition
05-35A/B	5

B. Tibia cultures - Resorption

Compound	IC50 in µM
GM6001	0.1 +/- 0.01
01-07A	> 30
02-07A	No Inhibition
02-08A	> 75
02-16A	No Inhibition
04-18A	No Inhibition
04-10A	> 75

Example 17 *The demineralised collagenolysis assay*

Inhibition of removal of collagen fibres in calvaria cultures

In order to investigate the roles of MMPs and cysteine proteinases in stimulated bone resorption, we cultured calvariae isolated from 5-day-old mice in the presence of 100 nM PTH, and in the absence or presence of various MMP inhibitors (the two hydroxamate-type inhibitors, RP59794 and CT1166; and the carboxylate-type inhibitor, CI-1) or cysteine proteinase inhibitors (E-64 or EST) for 1 day, and analysed the ultrastructure of the subosteoclastic resorption compartment. We made ultrathin sections of these calvariae, stained them with uranyl and lead, and examined them by electron microscopy. When the calvariae were cultured in the absence of a proteinase inhibitor, the front line of the ruffled border of the osteoclast was almost against the mineralised matrix, whereas a large amount of demineralised

collagen fibres were observed between the ruffled border of the osteoclast and the mineralised matrix in calvariae cultured in the presence of either an MMP inhibitor or a cysteine proteinase inhibitor, thereby indicating that the bone could be demineralised to some extent, but that collagen degradation was impaired by both types of proteinase inhibitor. In order to quantify this effect, pictures were taken and the surface of demineralised bone matrix between osteoclasts and mineralised bone was measured using a computerised X-Y tablet. The areas of demineralised matrix were 5 to 7 times larger in the calvariae treated with a proteinase inhibitor than in the controls, the effects being most dramatic and arising fastest when cysteine proteinases were inhibited. These data shows that in PTH-stimulated calvariae, not only cysteine proteinases but also metalloproteinases play a rate limiting role in the solubilisation of bone matrix by osteoclasts.

In the same cultures, it was interesting to notice that the areas of demineralised matrix in those resorption pits from which the osteoclasts had disappeared were much larger when the calvariae were cultured in the presence of an MMP inhibitor than when cultured with a cysteine proteinase inhibitor. In order to investigate the proteolytic events occurring in the latter situation, we precultured the calvariae for 1 day with a cysteine proteinase inhibitor. Some cultures were stopped at this stage for determination by electron microscopy of the initial amount of demineralised collagen fibres in the calvariae, and the remaining cultured for a further 18 hours in the absence or presence of an MMP inhibitor or a cysteine proteinase inhibitor, and then processed for electron microscopy. We saw that after the osteoclast had left its

resorption pit, bone lining cells occupied this pit and showed phagocytic activity towards the collagen remnants left by the osteoclast. During the 18-hour cultures performed in the absence of inhibitor or in the presence of a cysteine proteinase inhibitor, the areas of collagen remnants dropped to 20% of the initial values, whereas in the presence of an MMP inhibitor, this collagen degradation was almost completely inhibited.

Example 18 *The calvarial decalcification assay*

Inhibition of ^{45}Ca release from foetal mouse calvaria cultures.

^{45}Ca labelled calvariae from day 18 mouse foetuses were carefully isolated and subsequently cultured for 6 days in the absence or presence of 100 nM PTH, and in the absence or presence of either 10 μM of the hydroxamate-type MMP inhibitor, GM6001, or various concentrations of the novel phosphinate-type MMP inhibitor, 01-07A. Resorption was measured by the relative release of ^{45}Ca into the conditioned medium compared to the total amount of ^{45}Ca in the corresponding calvaria. The PTH-stimulated release of ^{45}Ca was inhibited dose-dependently by 01-07A (IC_{50}: approx. 2 μM) and to a level similar to that obtained by GM6001 (Fig. 20).

Example 19 *The tetracycline-labelled bone resorption assay*

Inhibition of bone resorption *in vivo*

In order to investigate the influence of MMP inhibitors on bone resorption *in vivo*, bones of newborn mice were labelled by

tetracycline. Briefly, pregnant mice were injected with 160 mCi ^{3}H-tetracycline 1 day before parturition. Between 3 and 6 days of age, half of the mice of each litter were injected s.c. twice a day with a test substance, the other half were injected with saline. The mice were sacrificed at the age of 7 days, their tibiae and femurs were isolated, the ^{3}H-tetracycline was released by demineralisation of the bones in acid, and the level of ^{3}H-tetracycline was determined. The test substances included the MMP inhibitor, RP59794 (200 µg bid), as well as some established inhibitors of bone resorption: the bisphophonates, clodronate (Cl_2MBP) and pamidronate (APD) (both at 100 µg bid), and the cysteine proteinase inhibitor, E-64 (100 µg bid). Tibiae and femurs of mice treated with the MMP inhibitor had significantly elevated contents of ^{3}H compared to the corresponding bones of the non-treated control group of mice, and as high levels of ^{3}H as the bisphosphonate and cysteine proteinase inhibitor treated groups (Fig. 21). This shows that, more ^{3}H-tetracycline was retained in the bones of mice treated with MMP inhibitors or established inhibitors of bone resorption, and that the rate of bone resorption was decreased by these treatments. We conclude that the inhibitory activity exerted by MMP inhibitors on bone resorption can also be detected in vivo.

Example 20 *The bone metastasis assay*

Inhibition of experimental bone metastasis

When breast cancer cells are inoculated intracardially into nude mice the cells will metastasise to bone and induce osteoclast-dependent osteolysis.

Briefly, the oestrogen-receptor negative breast cancer cell line MDA-MB-231 (MDA-231/P) characterised by a high metastatic potential was cultured in E-MEM supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and 5% (v/v) FCS. An in vivo selected bone-seeking subclone of the MDA-231/P cells was isolated in our laboratory from bone metastases in a nude mice 28 days after cardiac inoculation. This subclone is hereafter referred to as MDA-231/B. MDA-231/B cells were maintained for up to 6 passages under the same conditions as MDA-231/P. A suspension of 10^5 MDA-231/B cells in 0.1 ml were injected into the left cardiac ventricle of 4-week old female BALB/c-nu/nu mice (M&B, Denmark) at Day 0. Three days before (Day -3), a slow releasing pellet containing 0.72 mg of 17 ß-oestradiol (Innovative Research of America Inc., Florida, USA) had been implanted s.c. in each of the mice. The pellet was removed at day 7. X-ray inspection was done at Day 19 and 28, and the radiographs of mice were captured by a video camera and projected to a computer monitor, where areas of osteolytic metastases in limbs and pelvis were measured using the stereological program CastGrid (Olympus A/S, Denmark).

The mice were usually sacrificed at Day 28, and the tibiae and femurs were isolated and fixed in 4% formaldehyde (4°C for 48 hrs), before decalcification in 15% EDTA-solution, pH 7.4, for 3 weeks. Five-mm sections of paraffin embedded tibiae or femurs placed on Superfrost PLUS slides (Menzel-Gläser, Germany) were air-dried overnight at 37°C. Slides were deparaffinised in two changes of toluene and rehydrated in decreasing concentrations of methanol. The demonstration of tartrate resistant acid phosphatase (TRAP) positive cells (i.e., osteoclasts) in bone sections was performed by incubation of the slides for 2 hrs in 0.7% (w/v) sodium acetate, 0.05% Naphtol-AS-

BI-phosphate, 0.74% (w/v) 5,5-diethyl-barbituric-acid, 0.23% (w/v) di-sodium tartrate, 0.16% (w/v) $NaNO_2$, 0.16% (w/v) pararosaniline. The slides were subsequently rinsed in running tap water for 10 min before counterstaining with Mayers haematoxylin for 1 min. Finally, the slides were rinsed in running tap water for 10 min, dehydrated in increasing concentrations of ethanol and toluene and coversliped with DPX Mountant (Fluka, Neu-Ulm, Switzerland).

In one experiment, the nude mice, which at Day 19 had osteolytic lesions according to radiography, were randomly divided into two groups. The mice of the two groups were i.p. injected daily for 10 consecutive days (Day 19-28) with vehicle or the hydroxamate-type MMP inhibitor, BB-94 (60 mg/kg) and then X-rayed again at Day 28. At Day 28, the area of tumour lesions in both groups had increased significantly from the equal levels at Day 19, but the increase was significantly higher in vehicle than BB-94 treated mice (Fig. 22). When sections of areas with osteolytic metastases were inspected histologically and compared for vehicle and BB-94 treated mice, vascular damage manifested by central haemorrhages in disintegrating tumours and areas of necrosis were observed for the BB-94 treated mice. Moreover, bone surfaces near these necrotic areas lacked the normal appearance of numerous actively resorbing osteoclasts.

In a second experiment, the nude mice were randomised three days before cancer cell inoculation (Day -3) to receive either vehicle for 32 days (until Day 28) or GM6001 (100 mg/kg weight) for either 11 days (until Day 7) or 32 days (until Day 28) by i.p. injections once daily. The GM6001 was well tolerated by the animals as judged by the animal weight curves (Fig 23A). The number (data not shown) and area (Fig. 23B) of osteolytic lesions was significantly higher in the vehicle treated group

than in the group treated with GM6001 group for 33 days, and a clear but non-significant reduction was also observed in the group treated with GM6001 for 11 days (Fig. 23B).

In a third experiment, the nude mice were randomised three days before cancer cell inoculation (Day -3) to receive either vehicle or GM6001 (100 mg/kg weight) by i.p. injections once daily for a total of up to 53 days (Day -3 to 49). The end-point measurement was animal survival. A highly significant increase in survival time was observed for the GM6001 treated mice (Fig. 24).

Example 21 *The osteoblast persistence assay*

Persistence of osteoblasts in 3-dimensional collagen gels

Mouse osteoblasts were seeded in a 3-dimensional collagen gel by preparation of a single cell suspension of $2,5*10^5$ MC3T3-E1 cells per ml in solubilised collagen (Nitta, Japan, 2.4 mg/ml of type I collagen at 4°C). The collagen was gelified in 48-well culture plates (Costar, The Netherlands, 0.3 ml per 0.8 cm^2 well at 37°C) and the cultures continued for 6 days by addition of 0.4 ml/well of α-MEM containing 5% FCS. The osteoblasts were cultured in the absence or presence of 10 μM of inhibitors of the different classes of proteolytic enzymes: the serine proteinase inhibitor, aprotinin; the cysteine proteinase inhibitor, E-64; the aspartic proteinase inhibitor, pepstatin; and the hydroxamate-type matrix metalloproteinase inhibitor GM6001. The number of viable osteoblasts at Day 6 was assessed by staining with AlamarBlue™ (Accumed, USA) (Fig. 25).

To clarify if the observed reduction in osteoblast number was a general feature of MMP inhibitors or restricted to

hydroxamates, the novel phosphinate based MMP inhibitor 01-07A was compared to GM6001 and another well characterised hydroxamate-type MMP inhibitor, BB-94. Furthermore, to ensure that the previously observed GM6001-induced reduction in osteoblast number was not limited to the cell line MC3T3-E1, the experiment was also performed in a primary osteoblast culture.

Primary osteoblasts were obtained from mouse calvariae from 18-day-old foetuses. After 1 passage, cells were seeded at $1*10^5$ cells per ml collagen suspension as described above and cultured for 4 to 6 days in the absence or presence of 10 μM MMP inhibitor. The reduction in osteoblast numbers induced by the two hydroxamate inhibitors, GM6001 and BB94, was not mimicked by the phosphinate-type inhibitor 01-07A (Fig. 26). Similar results were obtained in a dose-response experiment, when using MC3T3-E1 cells instead of primary osteoblasts (Fig. 27). Thus, the adverse effect on osteoblast number observed for hydroxamate-type MMP inhibitors are not observed for a phosphinate-type MMP inhibitor or inhibitors of non-MMP proteinases.

Example 22 *The in vitro bone formation assay*

Osteoblast differentiation and bone formation *in vitro*

The influence of MMP inhibitors on cell differentiation and bone nodule formation was investigated in osteoblast cultures.

Differentiation of MC3T3-E1 osteoblasts was induced by addition of 50 µg/ml ascorbic acid and 10 mM β-glycerol-phosphate to α-MEM supplemented with 10% FCS. Cell

proliferation was monitored by staining with AlamarBlue™ (Accumed, USA). Osteoblast differentiation was assessed by quantification of alkaline phosphatase activity in the conditioned medium (a marker for the early phase of osteoblast differentiation) and by quantification of bone nodule formation according to Alizarin Red staining (a marker for the late phase of osteoblast differentiation). MC3T3-E1 cells were cultured for 21 days in the absence or presence of increasing concentrations of the hydroxamate-type MMP inhibitor, GM6001 (Fig. 28), or at a fixed concentration of 10 μM of either GM6001 or the novel phosphinate-type MMP inhibitor, 01-07A (Fig. 29). No significant effects of the MMP inhibitors were observed for osteoblast proliferation or early phase differentiation, (i.e., alkaline phosphatase activity) (data not shown), whereas GM6001 but not 01-07A caused a significant reduction in late phase differentiation (i.e., bone formation). Thus, the adverse effect on bone formation observed for the hydroxamate-type MMP inhibitor was not seen for the phosphinate-type MMP inhibitor.

References.

Bartlett PA, Marlowe CK. Phosphonamidates as transition-state analogue inhibitors of thermolysine. Biochem 22: 4618-4624. 1983.

Baylis EK, Campbell CD, Dingwall JG. 1-aminoalkyl-phosphonous acids. Part 1. Isosters of the protein amino acids. J Chem Soc Perkin Trans 1: 2845-2853. 1984.

Blankemeyer-Menge B, Nimitz M, Frank R. An efficient method for anchoring Fmoc-amino acids to hydroxyl-functionalized solid supports. Tetrahedron Lett 31: 1701-1704. 1990.

Blavier L, Delaissé JM. Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. J Cell Sci 108: 3649-3659. 1995.

Brown PD, Giavazzi R. Matrix metalloproteinase inhibition: a review of anti-tumour activity. Annals Oncol 6: 967-974. 1995.

Buchardt J, Meldal M. A chemically inert hydrophilic resin for solid phase organic synthesis. Tetrahedron Lett 39: 8695-8698. 1998.

Chen QJ, Lund L, Lenhard T, Engsig M, Winding B, Therkildsen B, Pedersen AC, Larsen D, Werb Z, Foged NT, Delaissé JM. MMP-

9 is a regulator of osteoclast recruitment as demonstrated by targeted mutagenesis. Bone 23 (Suppl.): S548 (SA086). 1998.

Everts V, Korper W, Jansen DC, Steinfort J, Lammerse I, Heera S, Docherty AJ, Beertsen W. Functional heterogeneity of osteoclasts: matrix metalloproteinases participate in osteoclastic resorption of calvarial bone but not in resorption of long bone. FASEB J 13: 1219-1230. 1999.

Holmes CP, Jones DG. Reagents for combinatorial organic synthesis: Development of a new o-nitrobenzyl photolabile linker for solid phase synthesis. J Org Chem 60: 2813-2819. 1995.

Klein L, Li QX, Donovan CA, Powell AE. Variation of resorption rates in vivo of various bones in immature rats. Bone Miner 8:169-175. 1990.

Meldal M, Svendsen I, Breddam K, Auzanneau FI. Portion-mixing peptide libraries of quenched fluorogenic substrates for complete subsite mapping of endoprotease specificity. Proc Natl Acad Sci 91:3314-3318. 1994.

Nagase H, Fields CG, Fields GB. Design an characterization of a fluorogenic substrate selectively hydrolysed by stromelysin 1 (matrix metalloproteinase-3). J Biol Chem 269: 20952-20957. 1994.

Renil M, Ferreras M, Delaissé JM, Foged NT, Meldal M. PEGA supports for combinatorial peptide synthesis and solid-phase enzymatic library assays. J Peptide Sci 4: 195-210. 1998.

Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P, von Figura K. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. Proc Natl Acad Sci USA 95:13453-13458. 1998.

Sato T, Foged NT, Delaissé JM. The migration of purified osteoclasts through collagen is inhibited by matrix metalloproteinase inhibitors. J. Bone Miner Res 13: 59-66. 1998.

Yiotakis A, Vassiliou S, Jiracek J, Dive V. Protection of the hydroxyphosphinyl function of phosphinic dipeptides by adamantyl. Application to the solid-phase synthesis of phosphinic peptides. J Org Chem 61: 6601-6605. 1996.

Buchardt et al, "Phosphinic Peptide Matrix Metalloproteinase - 9 Inhibitors by Solid-Phase Synthesis Using a Building Block Approach". Chem. Eur. J. 1999, 5, No.10, 2877-2884.

Cited patents and patent applications.

WO98/03516
Reiter LA. Phosphinate based inhibitors of matrix metalloproteinases. Pfizer Inc. Filed Jun. 30, 1997.

WO93/16118
Meldal MP. Polyethylene or polypropylene glycol containing polymer. Carlsberg A/S. Filed Feb. 13, 1992.

EP-A-0,611,756
Sohda T, Fujisawa Y, Yasuma T, Mizoguchi J, Kori M, Takizawa M. Alcohol or aldehyde derivatives as cathepsin L inhibitor and bone resorption inhibitor. Takeda Chemical Ind. Ltd. Filed Feb. 17, 1994.

US-A-5,579,700
Caldwell CG, Durette PL, Goulet JL, Hagmann WK, Sahoo P. Substituted phosphinic acid-containing peptidyl derivatves as antidegenerative agents. Merck & Co, Inc. Filed Nov. 9, 1995.

US-A-5,776,903
Dive V, Jiracek J, Yiotakis A. Peptide derivatives usable as zinc endopeptidase 24-15 inhibitors. Commisariat A l'Energie Atomique. Filed Jan. 24, 1996

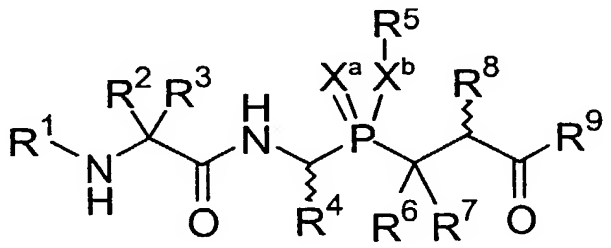
EP-A-0276436

Broadhurst MJ, Hando BK, Johnson WH, Lawton G, Machin PJ. Phosphinic acid derivatives. Hoffmann-La Roche. Filed Dec. 10, 1987.

5

CLAIMS

1. The use of a compound of the formula (I) or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of metabolic bone disease, where the compound of formula (I) is:



(I)

wherein

R^1 is

1. a hydrogen atom,
2. an amino-protecting group,
3. an alkyl, alkenyl, alkynyl or aryl group,
4. a group R^{1b}-NH-$CR^{1c}R^{1d}$-CO- in which
   - 4.1. R^{1b} is
     - 4.1.1. a hydrogen atom,
     - 4.1.2. a natural or unnatural α-amino acid, or a peptide consisting of the same,
     - 4.1.3. an alkyl, alkenyl, alkynyl or aryl group,
   - 4.2. R^{1c} and R^{1d} independently of each other are

4.2.1. a hydrogen atom,

4.2.2. a radical corresponding to a side chain of a natural and non-natural α-amino acid,

4.2.3. an alkyl, alkenyl, alkynyl or aryl group,

5. a group R^{1e}-CO- in which R^{1e} is

5.1. a hydrogen atom.

5.2. an alkyl, alkenyl, alkynyl or aryl group,

6. a group R^{1f}-SO_2- in which R^{1f} is an alkyl, alkenyl, alkynyl or aryl group.

R^2, R^3, R^4 and R^8 independently of each other are

1. a group as defined for R^{1c},

2. a group R^{2a}-CH_2- in which R^{2a} is

2.1. an aryl group, or

2.2. a heteroalicylic or heteroaromatic group,

R^5 is

1. a hydrogen atom, or

2. an alkyl, alkenyl, alkynyl or aryl group,

R^6 and R^7 independently of each other are

1. a hydrogen atom, or

2. an alkyl group,

R^9 is

1. a group R^{3a}-X^c- in which

1.1. R^{3a} is

1.1.1. an alkyl, alkenyl, alkynyl or aryl group,

1.1.2. a heteroalicylic or heteroaromatic group, or

1.1.3. a group as defined for R^2,

1.2. X^c is O, S or NH,

X^a and X^b are:

1. O
2. S
3. NH.

2. The use as claimed in Claim 1, wherein the compound of formula (I) or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a metalloproteinase.

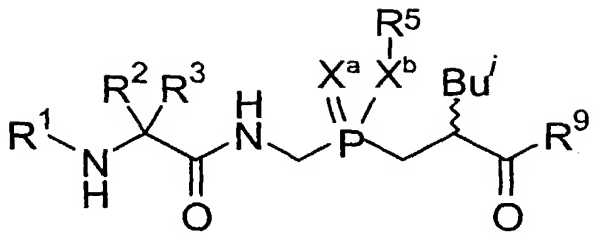
3. The use as claimed in Claim 2, wherein the compound of formula (I) or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a matrix metalloproteinase.

4. The use as claimed in Claim 1, wherein the compound of formula (I) or a pharmaceutically acceptable salt thereof in a concentration of 50 μM or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 50% compared to the appropriate vehicle treated control, one or more of the following activities: the osteoclast invasion in the *collagen invasion assay* or in the *bone lining cell invasion assay*, the osteoclastic pericellular collagenolysis or the distance of migration in

the pericellular collagenolysis assay, the TGF-β induced increase in accessible surface area of a culture of bone lining cells, or the osteoclastic bone resorption induced by treatment with TGF-β of a bone lining cell layer seeded on a bone substratum, or the TGF-β induced decalcification of cultured foetal mouse calvariae in the *bone lining cell elongation assay*, the decalcification or the number of invading osteoclast in a culture of foetal mouse metatarsals in the *metatarsal assay*, the removal in calvarial cultures of demineralised collagen fibres by osteoclasts in the subosteoclastic resorption zone or by bone lining cells in the resorption pits left by the osteoclasts in *the demineralised collagenolysis assay*, or the release of ^{45}Ca in *the calvarial decalcification assay*, or in a daily dose of 100 mg/kg or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 20% compared to the appropriate vehicle treated control, one or more of the following activities: the release of ^{3}H in the *tetracycline-labelled bone resorption assay*, or the number, area or mortality rate in the *bone metastasis assay*.

5. The use as claimed in Claim 1, wherein the compound of formula (I) has a K_i-value of 100 nM or less with one or more of the MMPs, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 or MMP-20, and a K_i-value at least 100 times higher than the lowest observed K_i-value for an MMP with two or more of the MMPs, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14 or MMP-20.

6. A compound of the formula (II):



(II)

or a pharmaceutically acceptable salt thereof, wherein: R^1, R^2, R^3, R^5, R^9, and X^a and X^b are as defined in claim 1.

7. A compound as claimed in Claim 6, or a pharmaceutically acceptable salt thereof for use in a method of medical treatment by therapy or prophylaxis.

8. The use of a compound of claim 6 or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of metabolic bone disease.

9. A compound as claimed in Claim 6 or Claim 7 or the use thereof as claimed in Claim 8, wherein the compound of formula (II) or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a metalloproteinase.

10. A compound as claimed in Claim 6 or Claim 7 or the use thereof as claimed in Claim 8, wherein in the compound of formula (II) or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a matrix metalloproteinase.

11. A compound as claimed in Claim 6 or Claim 7 or the use thereof as claimed in Claim 8, wherein the compound of formula (II) or a pharmaceutically acceptable salt thereof in a concentration of 50 μM or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 50% compared to the appropriate vehicle treated control, one or more of the following activities: the osteoclast invasion in the *collagen invasion assay* or in the *bone lining cell invasion assay*, the osteoclastic pericellular collagenolysis or the distance of migration in *the pericellular collagenolysis assay*, the TGF-β induced increase in accessible surface area of a culture of bone lining cells, or the osteoclastic bone resorption induced by treatment with TGF-β of a bone lining cell layer seeded on a bone substratum, or the TGF-β induced decalcification of cultured foetal mouse calvariae in the *bone lining cell elongation assay*, the decalcification or the number of invading osteoclast in a culture of foetal mouse metatarsals in the *metatarsal assay*, the removal in calvarial cultures of demineralised collagen fibres by osteoclasts in the subosteoclastic resorption zone or by bone lining cells in the resorption pits left by the osteoclasts in *the demineralised collagenolysis assay*, or the

release of ^{45}Ca in *the calvarial decalcification assay*, or in a daily dose of 100 mg/kg or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 20% compared to the appropriate vehicle treated control, one or more of the following activities: the release of ^{3}H in the *tetracycline-labelled bone resorption assay*, or the number, area or mortality rate in the *bone metastasis assay*.

12. A compound as claimed in Claim 6 or Claim 7 or the use thereof as claimed in Claim 8, wherein the compound of formula (II) has a K_i-value of 100 nM or less with one or more of the MMPs, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 or MMP-20, and a K_i-value at least 100 times higher than the lowest observed K_i-value for an MMP with two or more of the MMPs, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14 or MMP-20.

13. A compound having or comprising a sequence described in Table 10 or a pharmaceutically acceptable salt thereof, or a sequence related thereto by the substitution of one or more amino acids without decreasing the effectiveness or without decreasing the selectivity of the compound as a metalloproteinase inhibitor, or a compound acting as a molecular mimic of any such compound in interacting with a metalloproteinase:

Table 10: Selected phosphinate based peptide derivatives

Phosphinate based peptide derivative	P5	P4	P3	P2	$G^{pc}L$	P2'	P3'	P4'	P5'
01-01		V	A	Y		K	S	R	G
01-02			A	Y		K	S	G	
01-03			V	Y		R	S	G	
01-04		G	L	A		W	L	P	G
01-05			L	A		W	L	G	
01-06			L	A		Q	L	G	
01-07	A	G	P	L		Y	A	R	G
02-01		L	M	Y		Y	A	P	G
02-02		I	M	Y		Y	A	P	G
02-03		K	M	Y		Y	A	P	G
02-04		Z	M	Y		Y	A	P	G
02-21		Q	M	Y		Y	A	P	G
02-05		L	L	Y		Y	A	P	G
02-06		L	I	Y		Y	A	P	G
02-07		L	P	Y		Y	A	P	G
02-08		L	M	R		Y	A	P	G
02-09		L	M	F		Y	A	P	G
02-10		L	M	Y		L	A	P	G
02-11		L	M	Y		I	A	P	G
02-12		L	M	Y		J	A	P	G
02-13		L	M	Y		Y	L	P	G
02-14		L	M	Y		Y	I	P	G
02-15		L	M	Y		Y	J	P	G
02-16		L	M	Y		Y	M	P	G
02-17		L	M	Y		Y	Y	P	G
02-22		L	M	Y		Y	W	P	G
02-18		L	M	Y		Y	A	L	G
02-19		L	M	Y		Y	A	I	G
02-20		L	M	Y		Y	A	J	G
04-01				Y		Y			
04-19				Y		Y	W		
04-20				Y		Y	M		
04-21			M	Y		Y			
04-18		I	L	F		L	M	I	G
04-03		T	L	Y		L	D	G	
04-04		V	L	Y		T	L	S	G

04-05	I	M	Y		V	K	F	G
04-07	T	L	Y		R	A	I	G
04-09	T	L	R		L	F	F	G
04-10	I	L	R		M	A	P	G
04-11	S	L	F		R	D	I	G
04-12	L	M	F		Y	L	S	G
04-13	I	M	Y		Y	M	T	G
04-14	K	F	Y		L	Y	A	G
04-15	Y	I	Y		T	M	P	G
04-16	S	M	A		Y	H	G	
04-17	I	M	R		L	S	E	G
04-02	I	L	L		N	L	I	G
04-06	L	I	E		R	K	G	
04-08	E	F	Y		K	Y	N	G
05-01	T	A	S		M	F	G	
05-02	M	Y	T		Y	K	L	G
05-03	T	R	K		S	E	L	G
05-04	T	R	Q		S	E	L	G
05-05		S	M		L	Y	A	G
05-06	L	A	A		Y	F	Y	G
05-07	E	S	N		Y	Y	G	
05-08	J	V	A		S	T	G	G
05-09	J	Y	M		L	Q	L	G
05-10	J	Y	M		L	K	L	G
05-11	V	F	K		M	A	K	G
05-12	N	R	A		F	Q	A	G
05-13	R	V	S		N	Y	G	G
05-14	G	J	K		Y	N	R	G
05-15	G	J	F		E	S	L	G
05-16	V	S	H		A	T	F	G
05-17	Y	P	E		S	A	S	G
05-18	J	M	V		L	Q	F	G
05-19	H	F	K		Q	G	F	G
05-20	Q	P	H		F	Y	D	G
05-21	S	J	D		G	V	E	G
05-22	R	J	D		T	L	J	G
05-23	R	P	P		L	L	G	
05-24	K	Y	F		G	P	M	G
05-25	G	M	G		P	F	L	G
05-26	T	N	P		N	V	E	G
05-27	G	T	V		A	K	Q	G
05-28		J	L		L	F	J	G
05-29	K	T	M		V	Q	L	G

05-30	K	T	M	V	K	L	G
05-31	Y	M	R	H	S	G	
05-32	N	V	V	Y	L	E	G
05-33	D	A	H	D	F	G	
05-34	T	P	L	E	A	D	G
05-35	A	P	A	L	A	Q	G
05-36	R	P	A	Q	M	R	G
05-37	Y	A	Y	K	Y	E	G
05-38	Y	A	Y	Q	Y	E	G
05-39	T	J	E	V	A	G	
05-40	V	A	K	Q	R	G	

$G^{PC}L$ is an abbreviation for -Glyψ(PO_2-CH_2)Leu-.

14. A compound as claimed in Claim 13, or a pharmaceutically acceptable salt thereof for use in a method of medical treatment by therapy or by prophylaxis.

15. The use of a compound of Claim 13 or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of metabolic bone disease.

16. A compound as claimed in Claim 13 or Claim 14 or the use as claimed in Claim 15, wherein the compound of Claim 13 or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a metalloproteinase.

17. A compound as claimed in Claim 13 or Claim 14 or the use as claimed in Claim 15, wherein the compound of Claim 13 or a pharmaceutically acceptable salt thereof acts by inhibition of the production or action of a matrix metalloproteinase.

18. A compound as claimed in Claim 13 or Claim 14 or the use as claimed in Claim 15, wherein the compound of Claim 13 or a pharmaceutically acceptable salt thereof in a concentration of 50 μM or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 50% compared to the appropriate vehicle treated control, one or more of the following activities: the osteoclast invasion in the *collagen invasion assay* or in the *bone lining cell invasion assay*, the osteoclastic pericellular collagenolysis or the distance of migration in *the pericellular collagenolysis assay*, the TGF-β induced increase in accessible surface area of a culture of bone lining cells, or the osteoclastic bone resorption induced by treatment with TGF-β of a bone lining cell layer seeded on a bone substratum, or the TGF-β induced decalcification of cultured foetal mouse calvariae in the *bone lining cell elongation assay*, the decalcification or the number of invading osteoclast in a culture of foetal mouse metatarsals in the *metatarsal assay*, the removal in calvarial cultures of demineralised collagen fibres by osteoclasts in the subosteoclastic resorption zone or by bone lining cells in the resorption pits left by the osteoclasts in *the demineralised collagenolysis assay*, or the release of ^{45}Ca in *the calvarial decalcification assay*, or in a daily dose of 100 mg/kg or less is able to reduce significantly ($p<0.05$ in the appropriate statistical test) and by more than 20% compared to the appropriate vehicle treated control, one or more of the following activities: the release of ^{3}H in the *tetracycline-*

labelled bone resorption assay, and the number, area or mortality rate in the *bone metastasis assay*.

19. A compound as claimed in Claim 13 or Claim 14 or the use as claimed in Claim 15, wherein the compound of claim 13 has a K_i-value of 100 nM or less with one or more of the MMPs, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14 or MMP-20, and a K_i-value at least 100 times higher than the lowest observed K_i-value for an MMP with two or more of the MMPs, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14 or MMP-20.

20. An anti-bone resorption agent comprising a compound of formula (I) operatively linked to a ligand targeting the compound of formula (I) to a proteinase involved in bone resorption or to the environment of the proteinase or incorporating such a ligand in or as one of the defined groups R^1, R^2, R^3, R^5 or R^9 of the formula.

21. An anti-bone resorption agent comprising a compound of Claim 6 operatively linked to a ligand targeting the compound of Claim 6 to a proteinase involved in bone resorption or to the environment of the proteinase or incorporating such a ligand in or as one of the defined groups R^1, R^2, R^3, R^5 or R^9 of the formula.

22. An anti-bone resorption agent comprising the compound of claim 13 operatively linked to a ligand targeting the compound

of claim 13 to a proteinase involved in bone resorption or to the environment of the proteinase.

23. A method for the synthesis of a compound of formula I which comprises carrying out a reaction to form a phosphorus to carbon bond between the phosphorus atom in a compound in which phosphorus bears at least the substituents

X^a, $-X^b$ $-R^5$, and $-CH(R^4)NH-$

and a carbon atom in a compound in which said carbon atom bears the substituents R^6, R^7 and $-C(R^8)-C(O)R^9$.

24. A method as claimed in Claim 23, wherein the said compound containing the carbon atom is attached to a solid support.

25. A method as claimed in Claim 24, comprising attaching said compound containing the carbon atom to said solid support by acylation by said compound of a primary amino group attached to said support.

26. A method as claimed in Claim 25, further comprising solid phase peptide synthesis on said support to provide said primary amino group.

27. A method as claimed in any one of Claims 24 to 26, further comprising solid phase peptide synthesis from said -NH- group attached to said $-CH(R^4)NH-$ substituent.

(12) INTERNATIONAL APPL[redacted]ION PUBLISHED UNDER THE PATENT C[redacted]RATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/25264 A3

(51) International Patent Classification[7]: C07K 7/02, 5/10, C07F 9/30, A61K 31/66, 38/07, 38/08, A61P 19/08

(21) International Application Number: PCT/EP00/09173

(22) International Filing Date:
19 September 2000 (19.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9922577.3 23 September 1999 (23.09.1999) GB

(71) Applicant *(for all designated States except US)*: **OSTEOPRO A/S** [DK/DK]; Herlev Hovedgade 207, DK-2730 Herlev (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BUCHARDT, Jens** [DK/DK]; Nr. Farmagsgade 69, 2th, DK-1364 Copenhagen K (DK). **FOGED, Niels, Tækker** [DK/DK]; Damvadvej 30, Søsum, DK-3670 Veksø (DK). **MELDAL, Morten** [DK/DK]; Nitivej 11, DK-2000 Frederiksberg (DK). **DELAISSE, Jean-Marie** [BE/DK]; Egebjerg Bygade 235, 1th, DK-2750 Ballerup (DK). **ENGSIG, Michael** [DK/DK]; Høgholtvej 34, DK-2720 Vanløse (DK). **FERRERAS, Mercedes** [ES/DK]; Jagtvej 201, DK-2100 Copenhagen Ø (DK). **KARSDAL, Morten** [DK/DK]; Ndr. Frihavnsgade 100A, DK-2100 Copenhagen Ø (DK). **OVEJERO, Maria del Carmen** [ES/DK]; Østerbrogade 118, DK-2100 Copenhagen Ø (DK). **SCHIØDT, Christine, Bruun** [DK/DK]; Risbyholmvej 20, DK-2700 Brønshøj (DK). **WINDING, Bent** [DK/DK]; Bartolinsgade 1, DK-1356 Copenhagen K (DK).

(74) Agent: **SMART, Peter, J.**; W.H. Beck, Greener & Co., 7 Stone Buildings, Lincoln's Inn, London WC2A 3SZ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

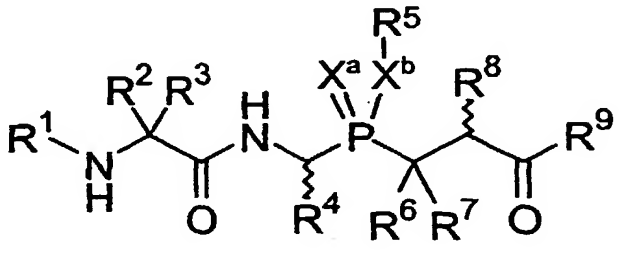
Published:
— *with international search report*

(88) Date of publication of the international search report:
18 October 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/25264 A3

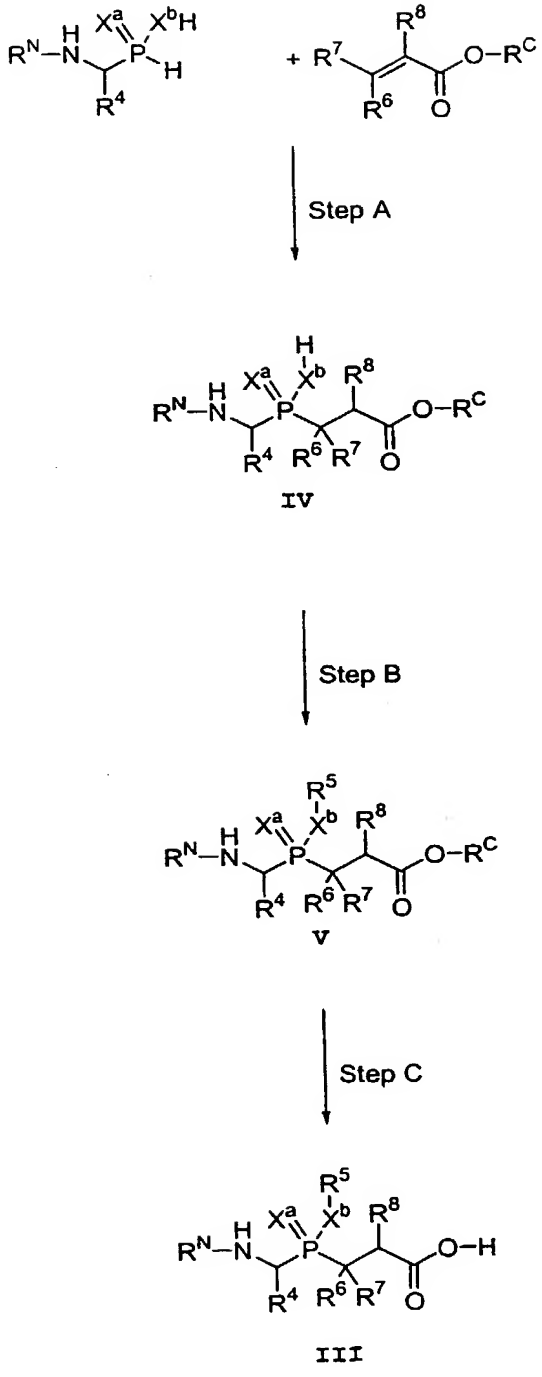
(54) Title: SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES



(I)

(57) Abstract: Compounds of Formula (I): wherein the substituents are as defined in the description and their use in the treatment of metabolic bone diseases such as osteoporosis and bone metastasis.

Figure 1



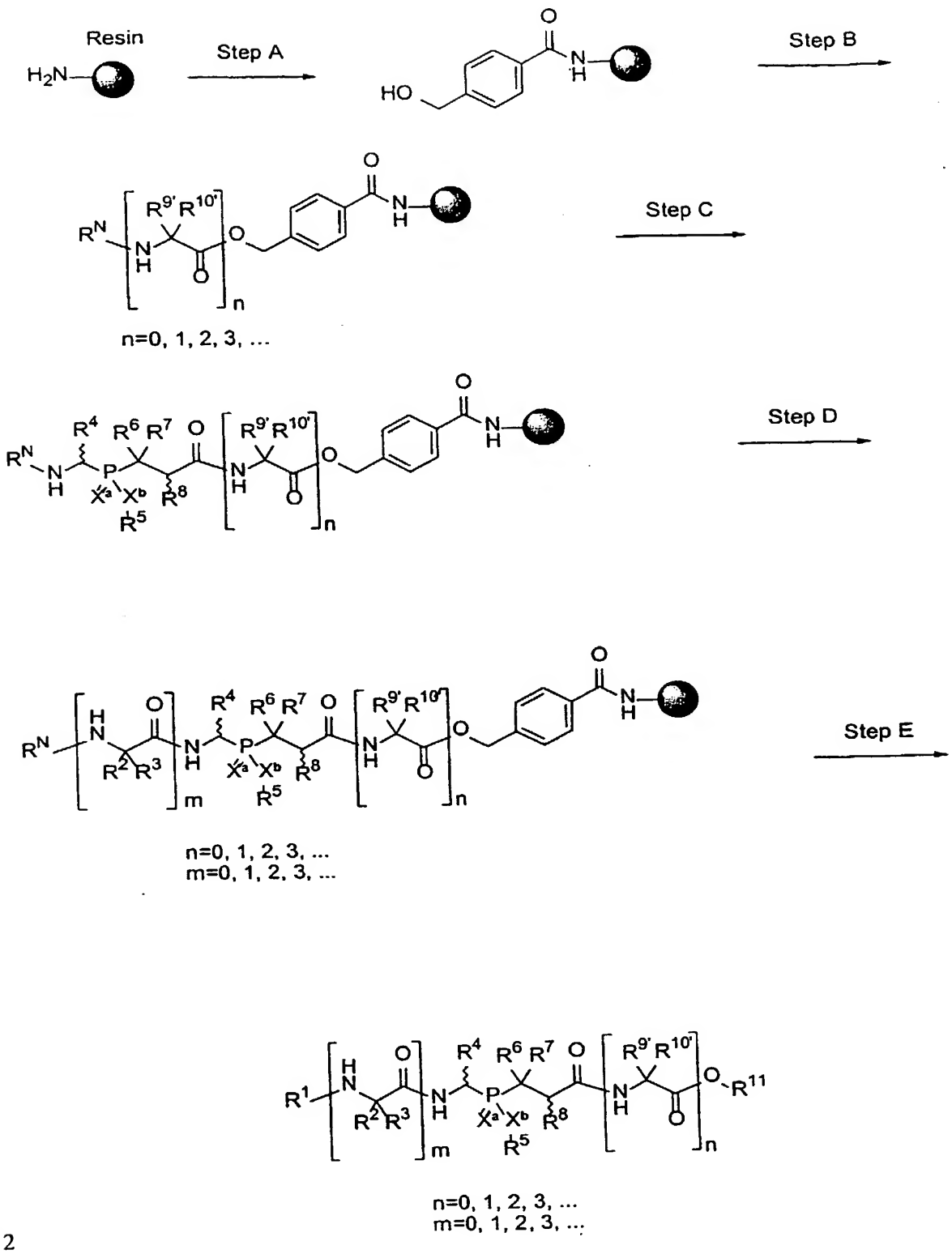


Figure 2

Fig. 3

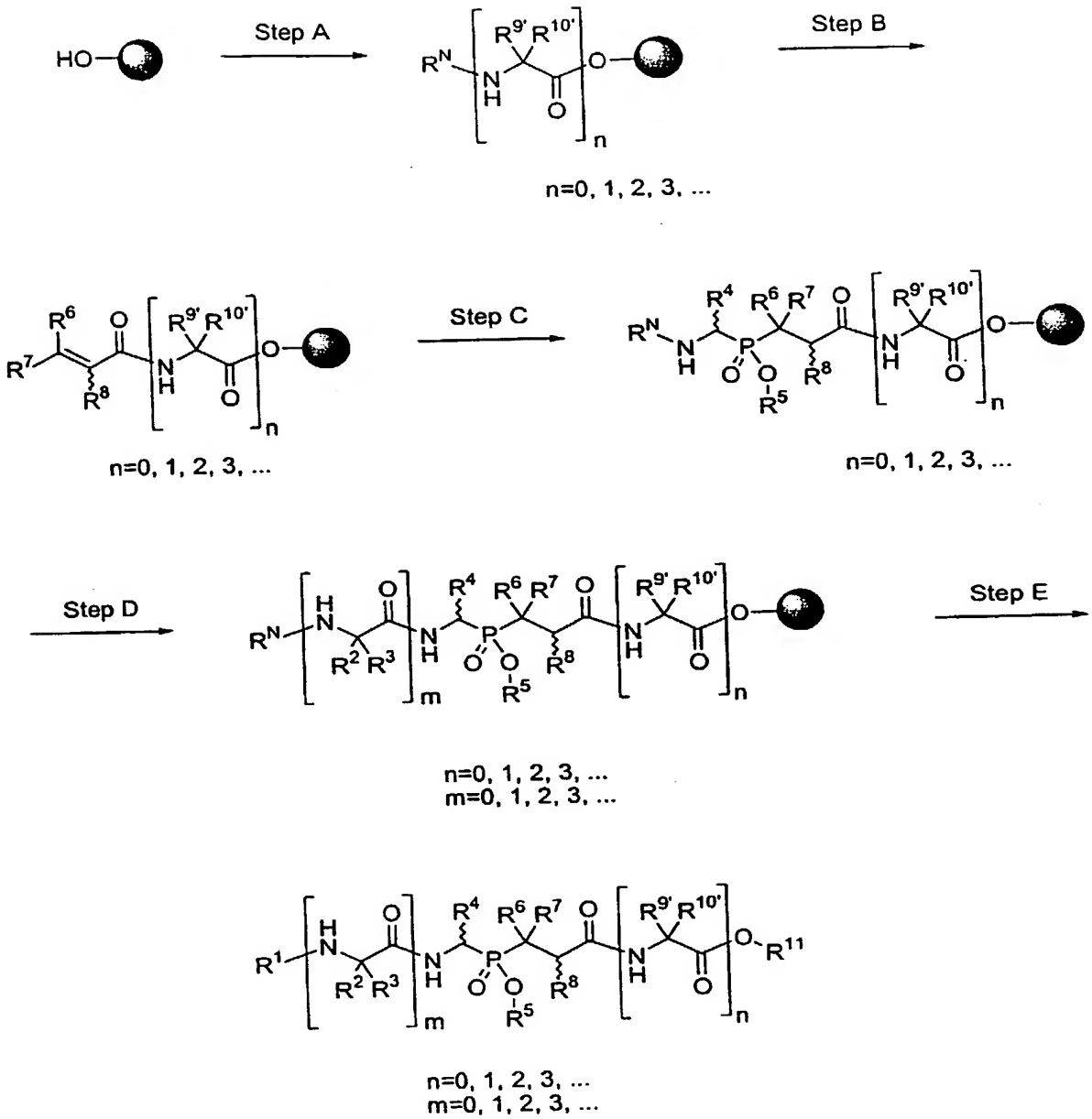

HO
Step A
Step B
n=0, 1, 2, 3, ...
Step C
n=0, 1, 2, 3, ...
n=0, 1, 2, 3, ...
Step D
Step E
n=0, 1, 2, 3, ...
m=0, 1, 2, 3, ...
n=0, 1, 2, 3, ...
m=0, 1, 2, 3, ...

Figure 4

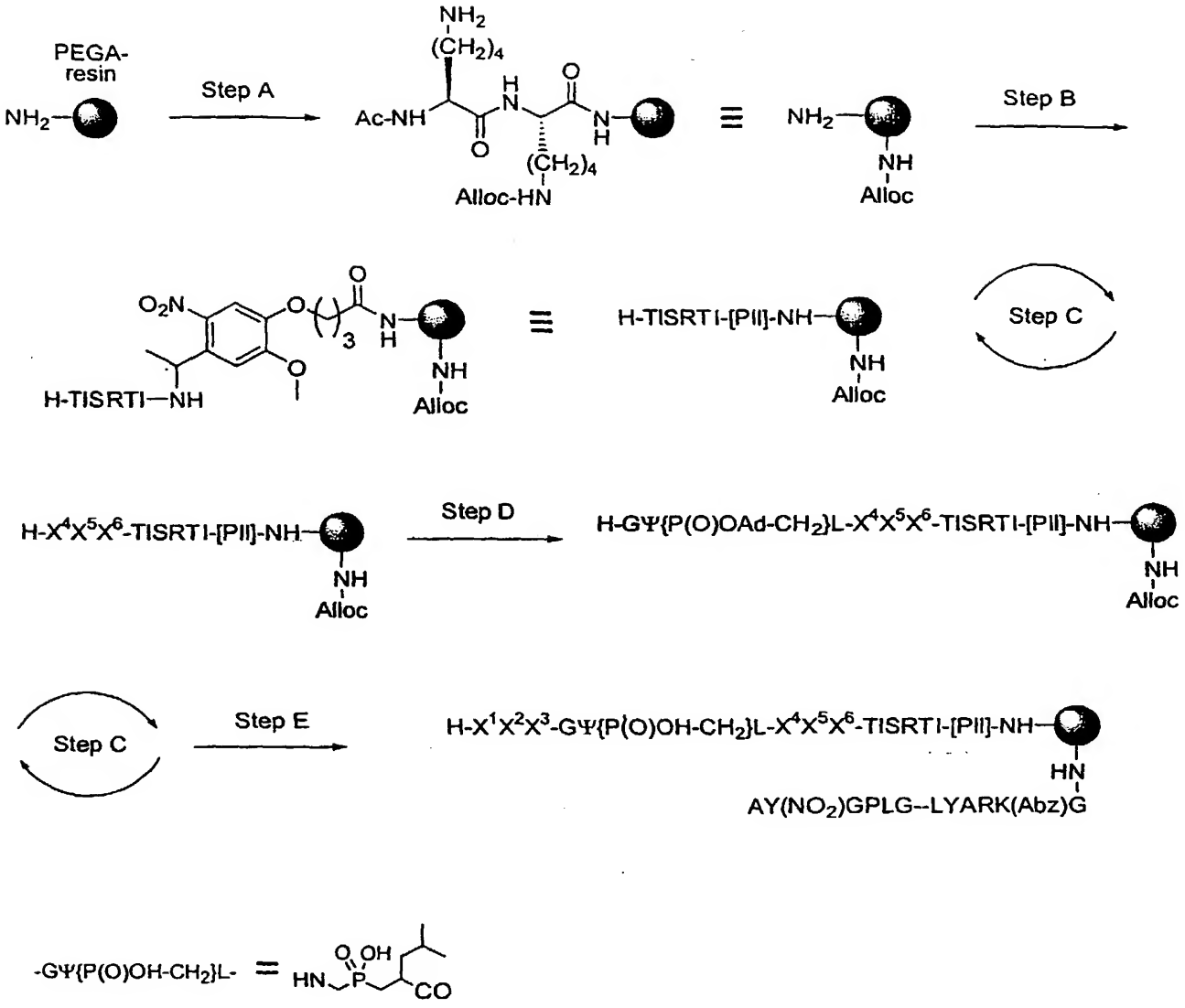

PEGA-resin
Step A
Step B
Step C
Step D
Step E
Step C
Alloc
Alloc-HN
H-TISRTI—NH
H-TISRTI-[PII]-NH
H-X⁴X⁵X⁶-TISRTI-[PII]-NH
H-GΨ{P(O)OAd-CH₂}L-X⁴X⁵X⁶-TISRTI-[PII]-NH
H-X¹X²X³-GΨ{P(O)OH-CH₂}L-X⁴X⁵X⁶-TISRTI-[PII]-NH
AY(NO₂)GPLG--LYARK(Abz)G
-GΨ{P(O)OH-CH₂}L-

Figure 5

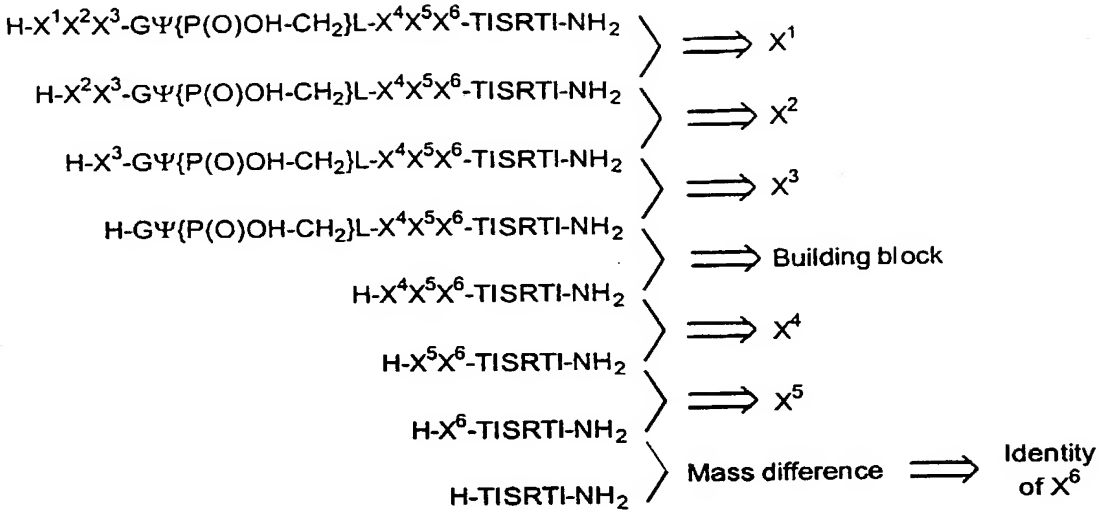
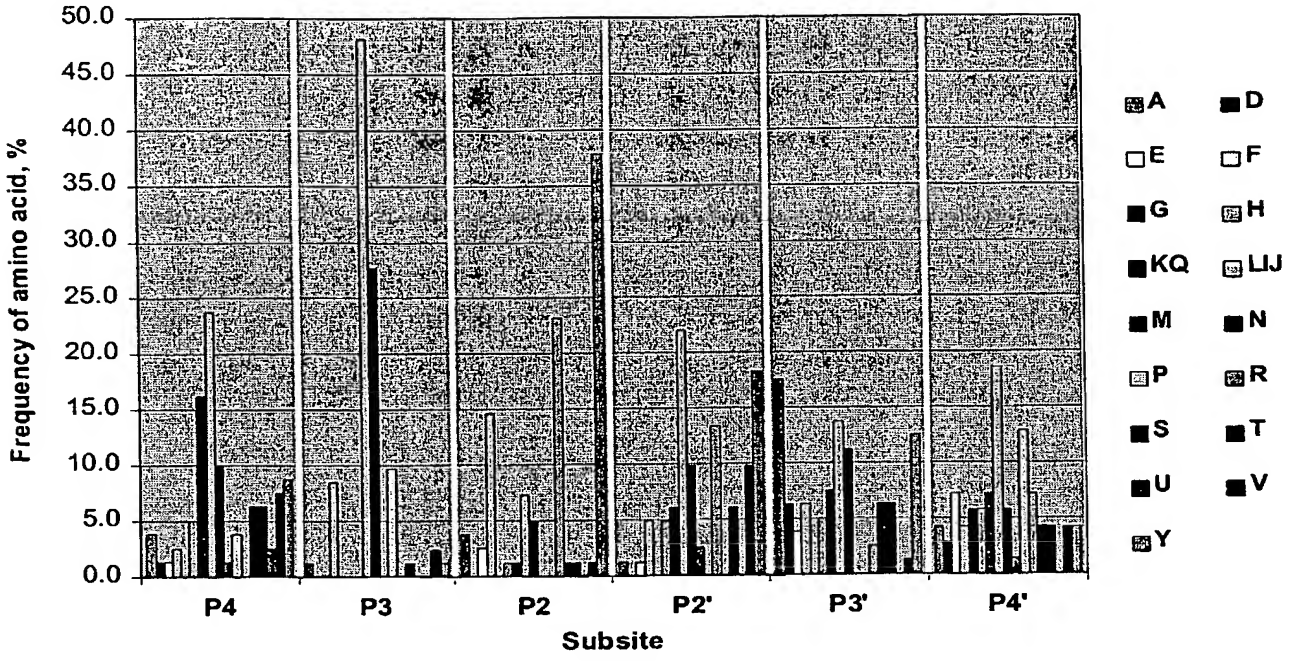

H-X1X2X3-GΨ{P(O)OH-CH2}L-X4X5X6-TISRTI-NH2
X1
H-X2X3-GΨ{P(O)OH-CH2}L-X4X5X6-TISRTI-NH2
X2
H-X3-GΨ{P(O)OH-CH2}L-X4X5X6-TISRTI-NH2
X3
H-GΨ{P(O)OH-CH2}L-X4X5X6-TISRTI-NH2
Building block
H-X4X5X6-TISRTI-NH2
X4
H-X5X6-TISRTI-NH2
X5
H-X6-TISRTI-NH2
Mass difference
Identity of X6
H-TISRTI-NH2

Figure 6


50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Frequency of amino acid, %
P4
P3
P2
P2'
P3'
P4'
Subsite
A
D
E
F
G
H
KQ
LIJ
M
N
P
R
S
T
U
V
Y

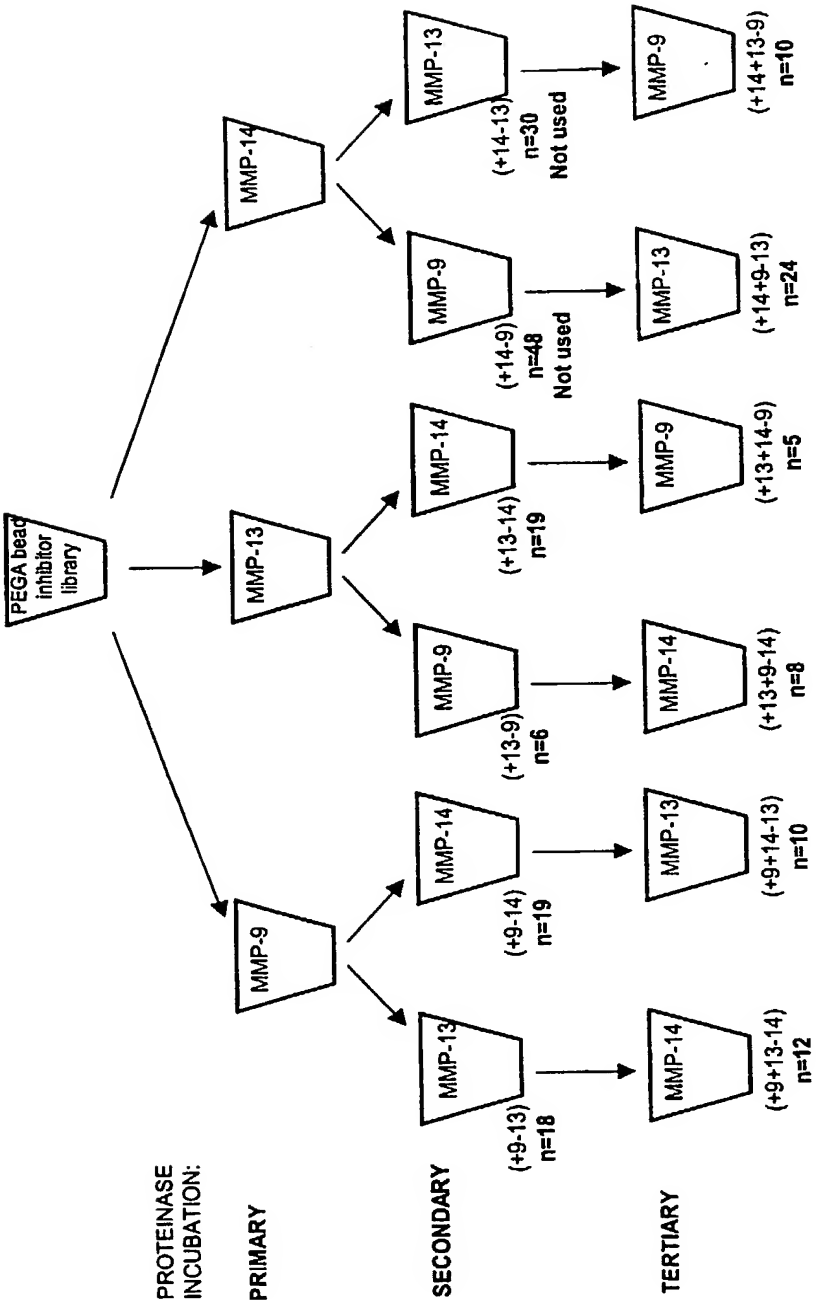

PEGA bead inhibitor library
PROTEINASE INCUBATION:
PRIMARY
SECONDARY
TERTIARY
MMP-9
MMP-13
MMP-14
(+9-13) n=18
(+9-14) n=19
(+13-9) n=6
(+13-14) n=19
(+14-9) n=48 Not used
(+14-13) n=30 Not used
(+9+13-14) n=12
(+9+14-13) n=10
(+13+9-14) n=8
(+13+14-9) n=5
(+14+9-13) n=24
(+14+13-9) n=10

Fig. 7

Figure 8

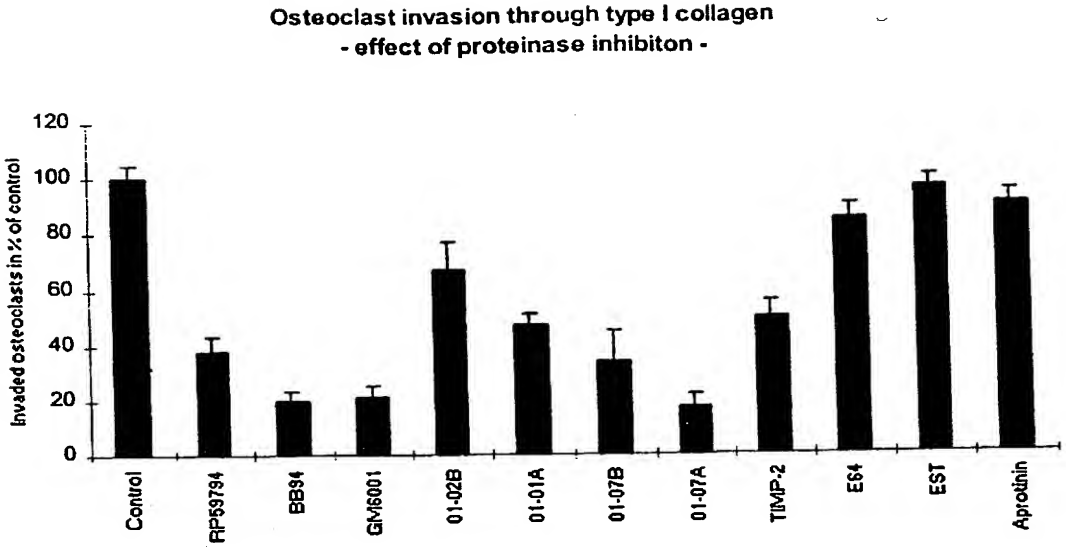

Osteoclast invasion through type I collagen
- effect of proteinase inhibiton -
Invaded osteoclasts in % of control
120
100
80
60
40
20
0
Control
RP59794
BB94
GM6001
01-02B
01-01A
01-07B
01-07A
TIMP-2
E64
EST
Aprotinin

Fig. 9

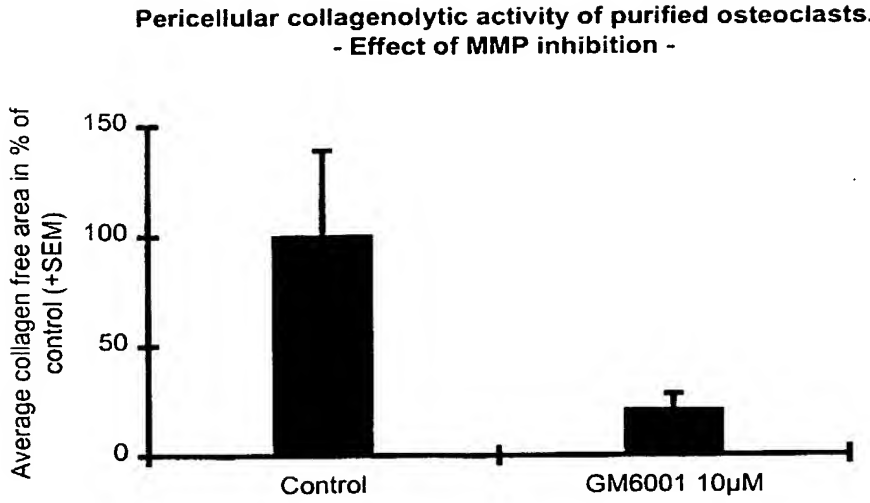

Pericellular collagenolytic activity of purified osteoclasts.
- Effect of MMP inhibition -
Average collagen free area in % of control (+SEM)
150
100
50
0
Control
GM6001 10µM

Fig. 10

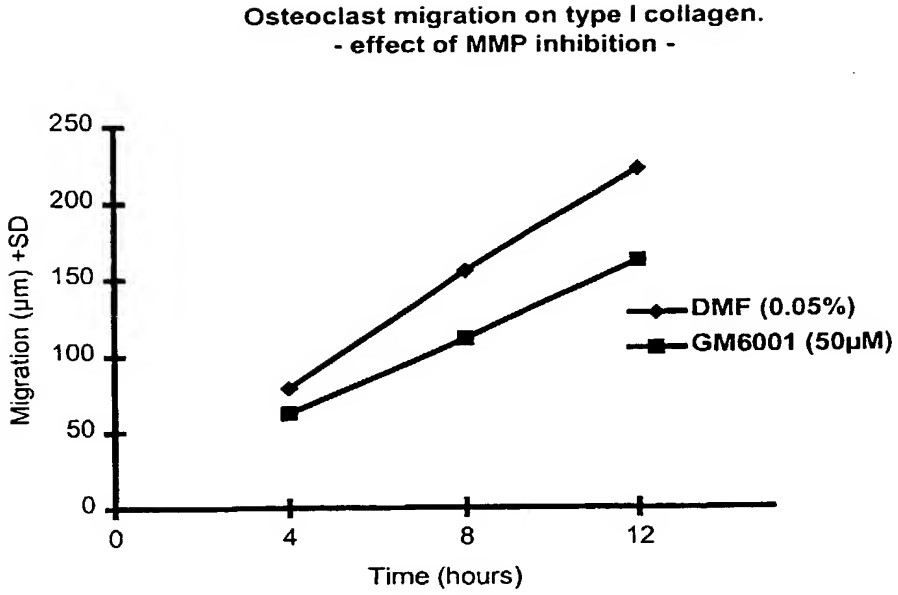

Osteoclast migration on type I collagen.
- effect of MMP inhibition -
Migration (µm) +SD
250
200
150
100
50
0
0
4
8
12
Time (hours)
DMF (0.05%)
GM6001 (50µM)

Fig. 11

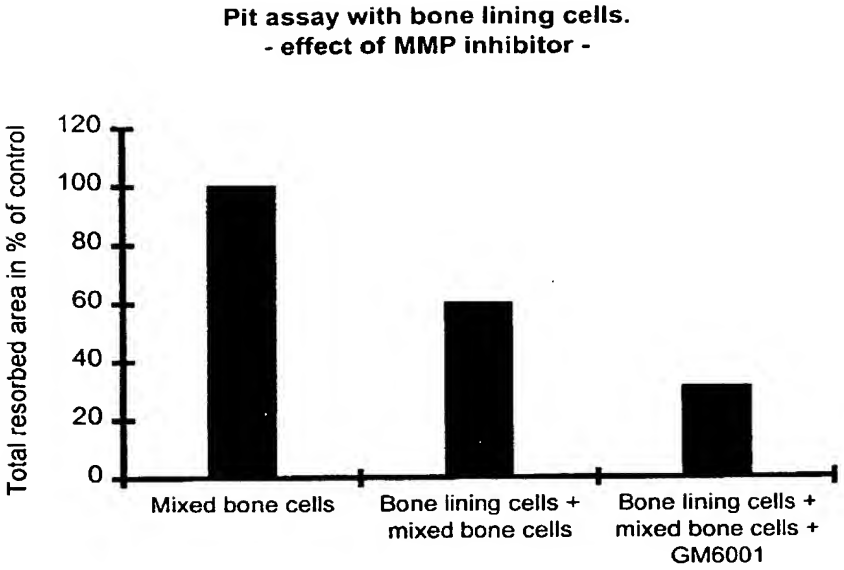

Pit assay with bone lining cells.
- effect of MMP inhibitor -
Total resorbed area in % of control
120
100
80
60
40
20
0
Mixed bone cells
Bone lining cells + mixed bone cells
Bone lining cells + mixed bone cells + GM6001

Fig. 12

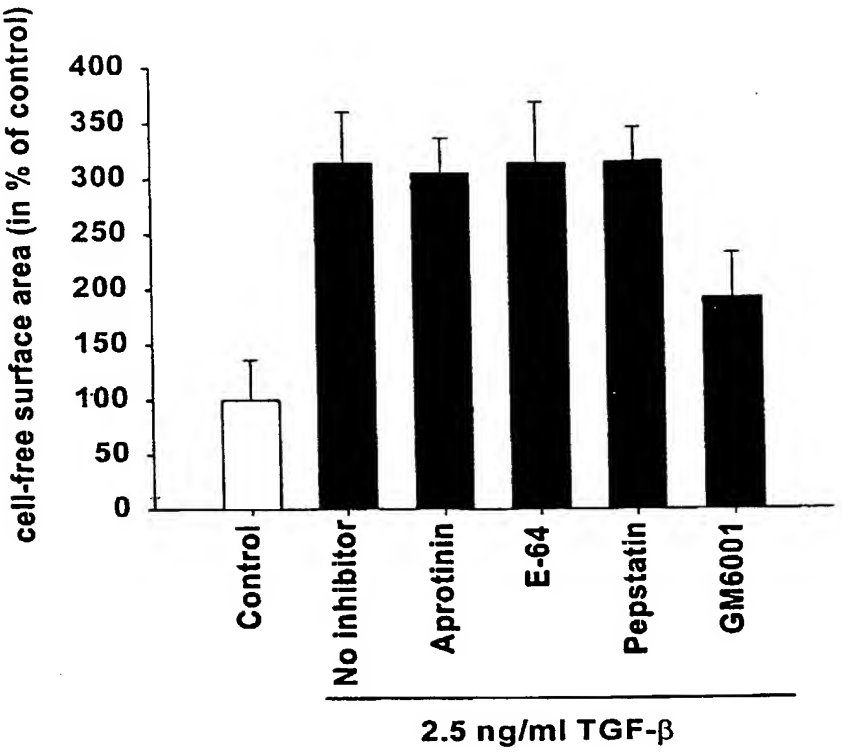

cell-free surface area (in % of control)
400
350
300
250
200
150
100
50
0
Control
No inhibitor
Aprotinin
E-64
Pepstatin
GM6001
2.5 ng/ml TGF-β

Fig. 24

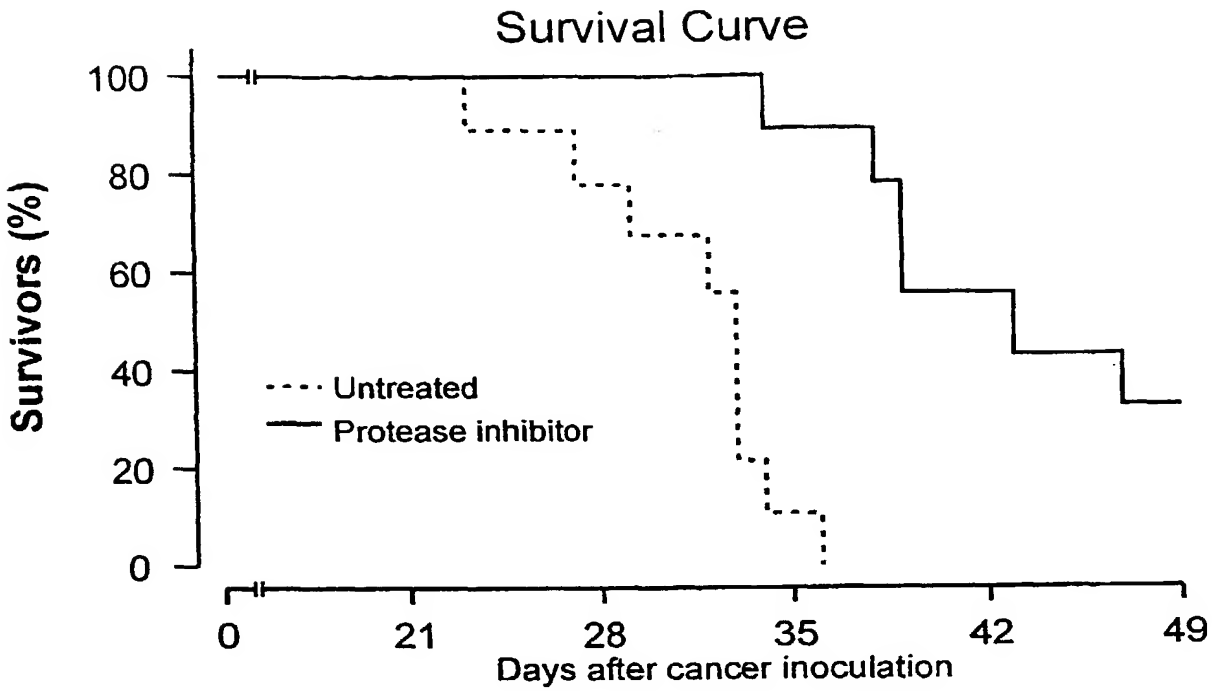
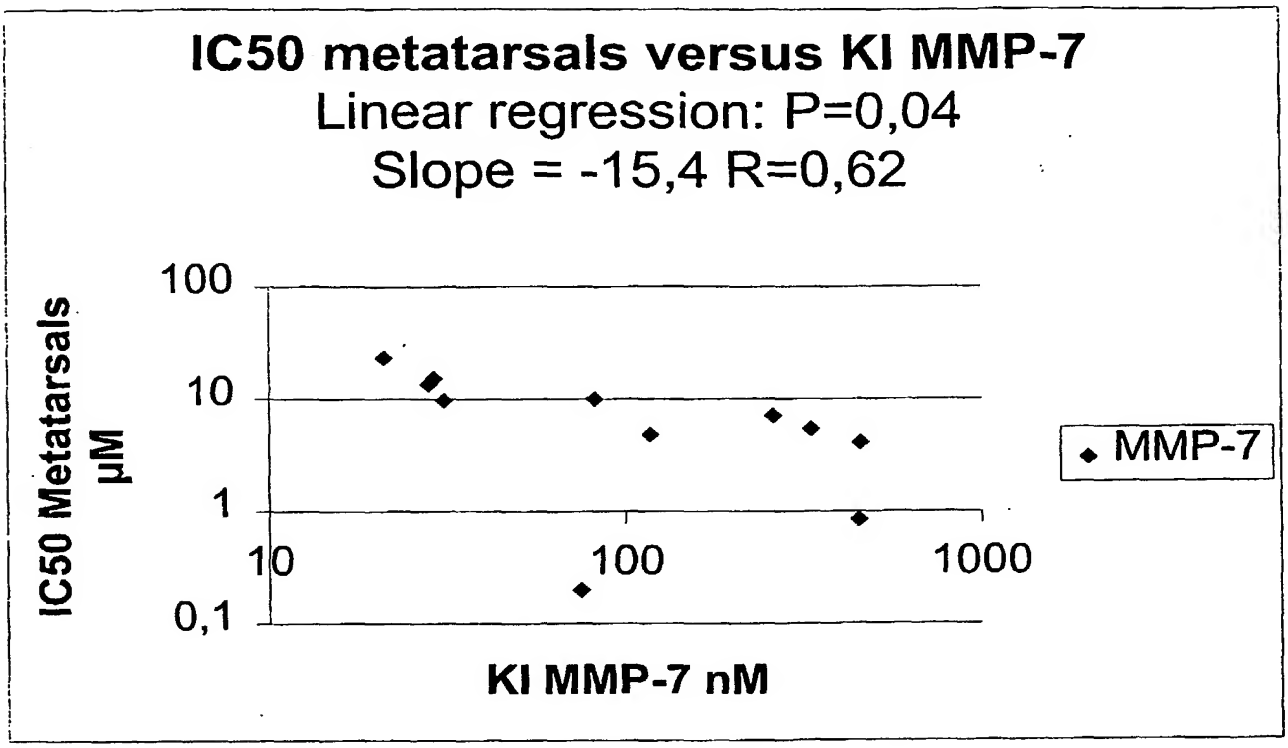

Survival Curve
Survivors (%)
100
80
60
40
20
0
Untreated
Protease inhibitor
0
21
28
35
42
49
Days after cancer inoculation

Fig 19

KI Value as predictor of IC50 value in metatarsals



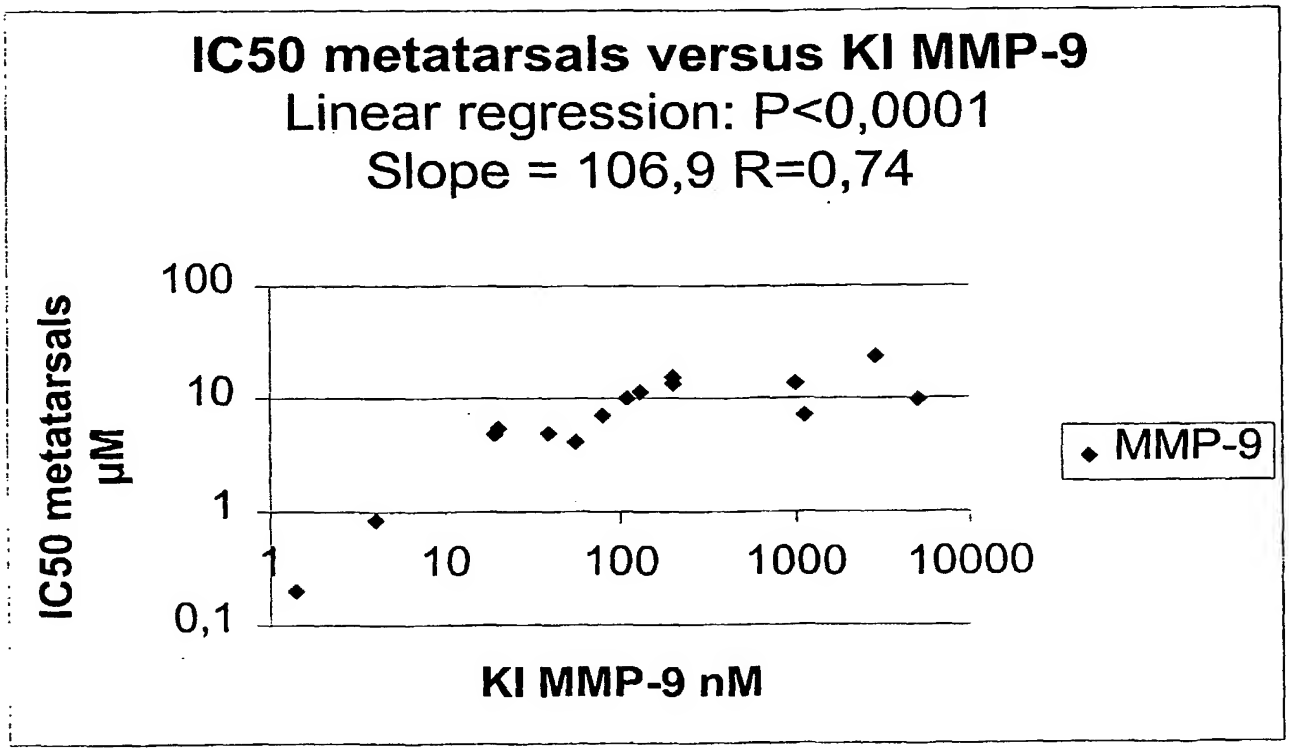
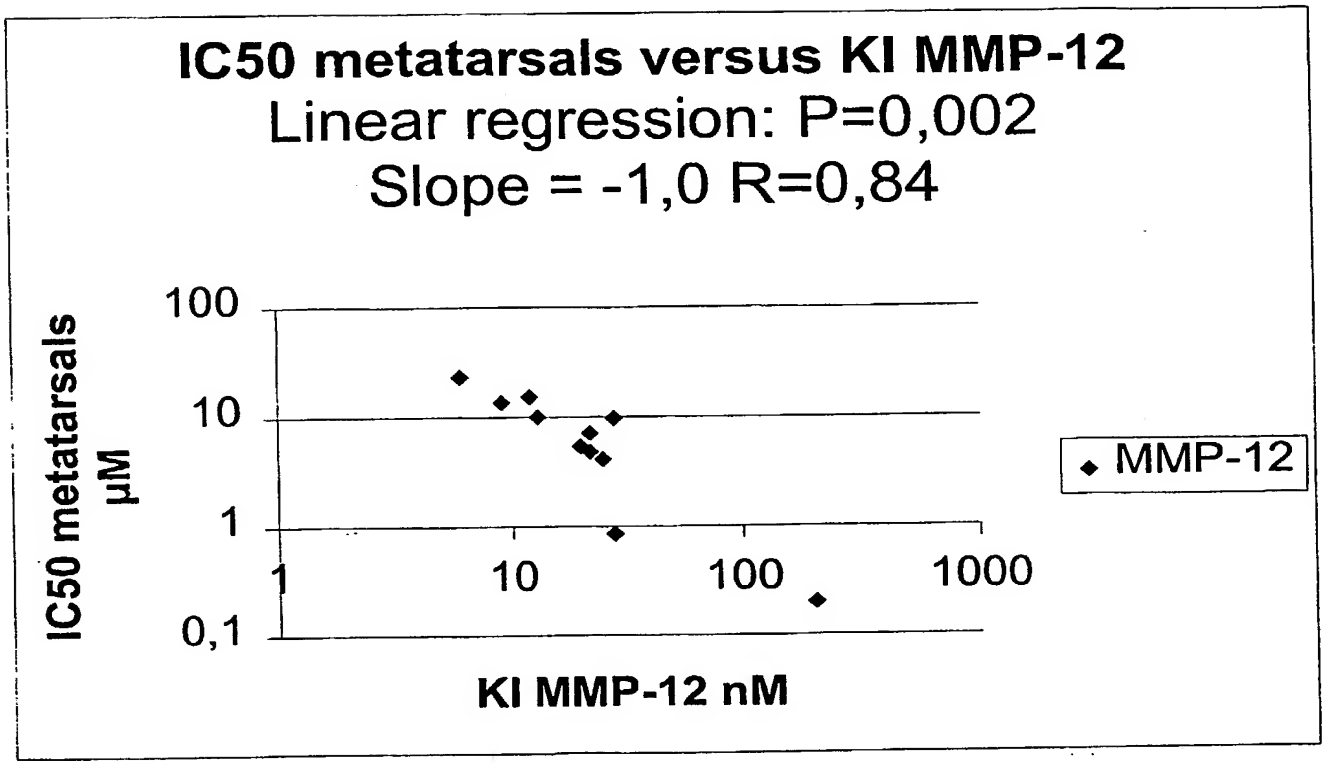


Fig 19/2


IC50 metatarsals versus KI MMP-12
Linear regression: P=0,002
Slope = -1,0 R=0,84
IC50 metatarsals µM
100
10
1
0,1
1
10
100
1000
MMP-12
KI MMP-12 nM

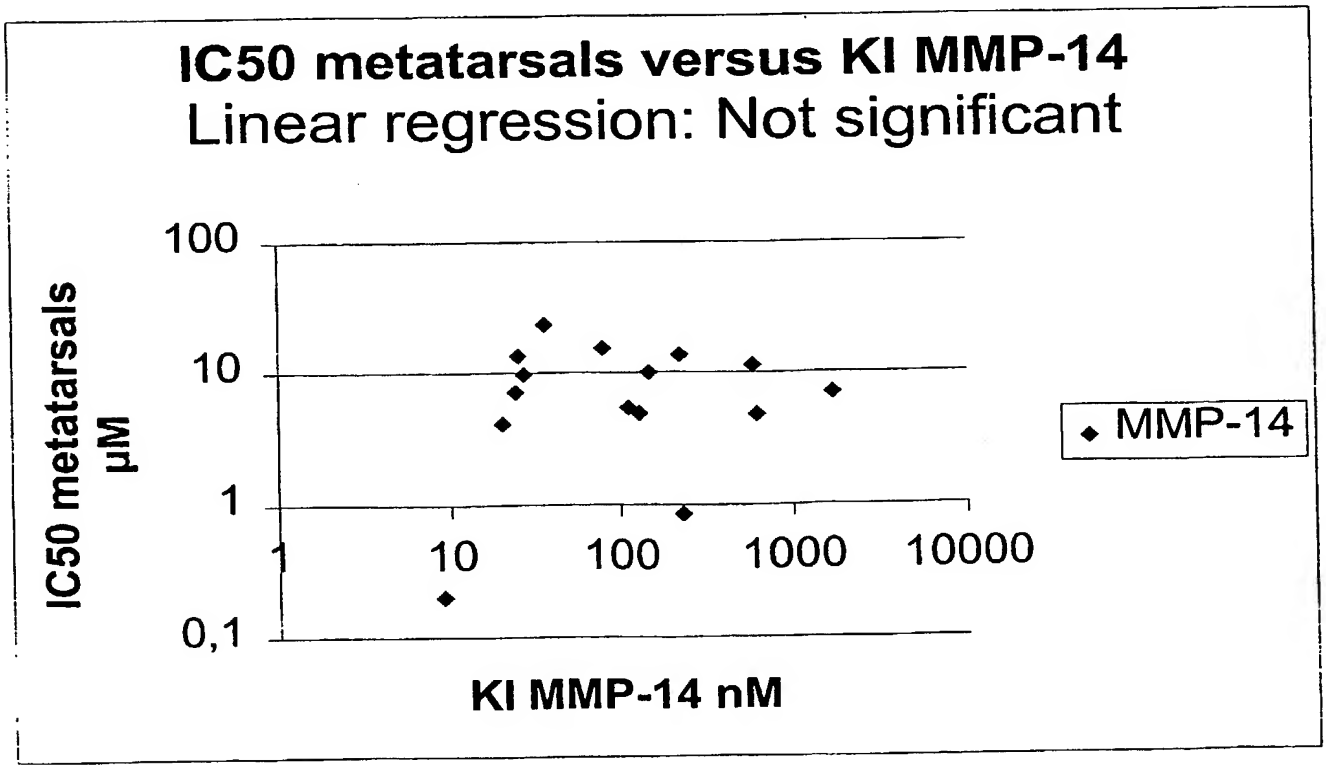
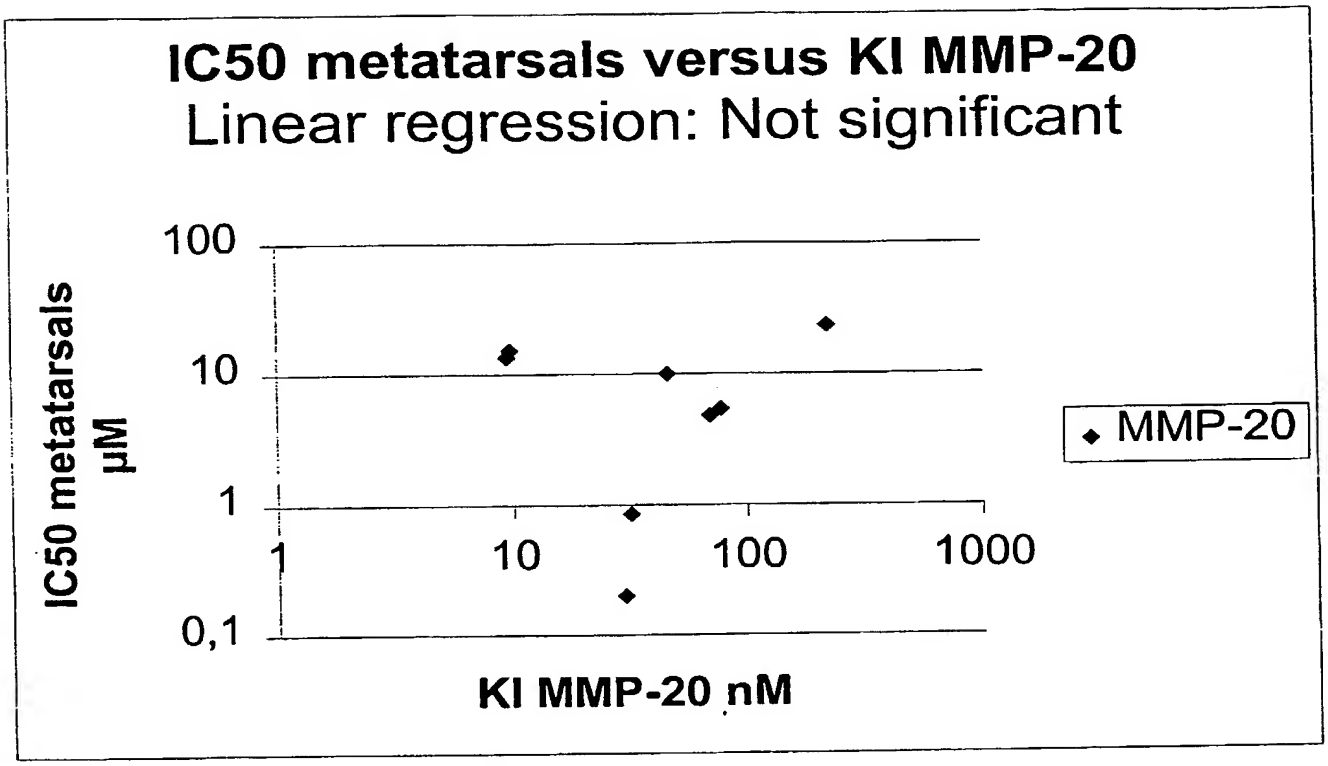

IC50 metatarsals versus KI MMP-14
Linear regression: Not significant
IC50 metatarsals µM
100
10
1
0,1
1
10
100
1000
10000
MMP-14
KI MMP-14 nM

Fig 19/3


IC50 metatarsals versus KI MMP-20
Linear regression: Not significant
100
10
1
0,1
IC50 metatarsals µM
1
10
100
1000
MMP-20
KI MMP-20 nM

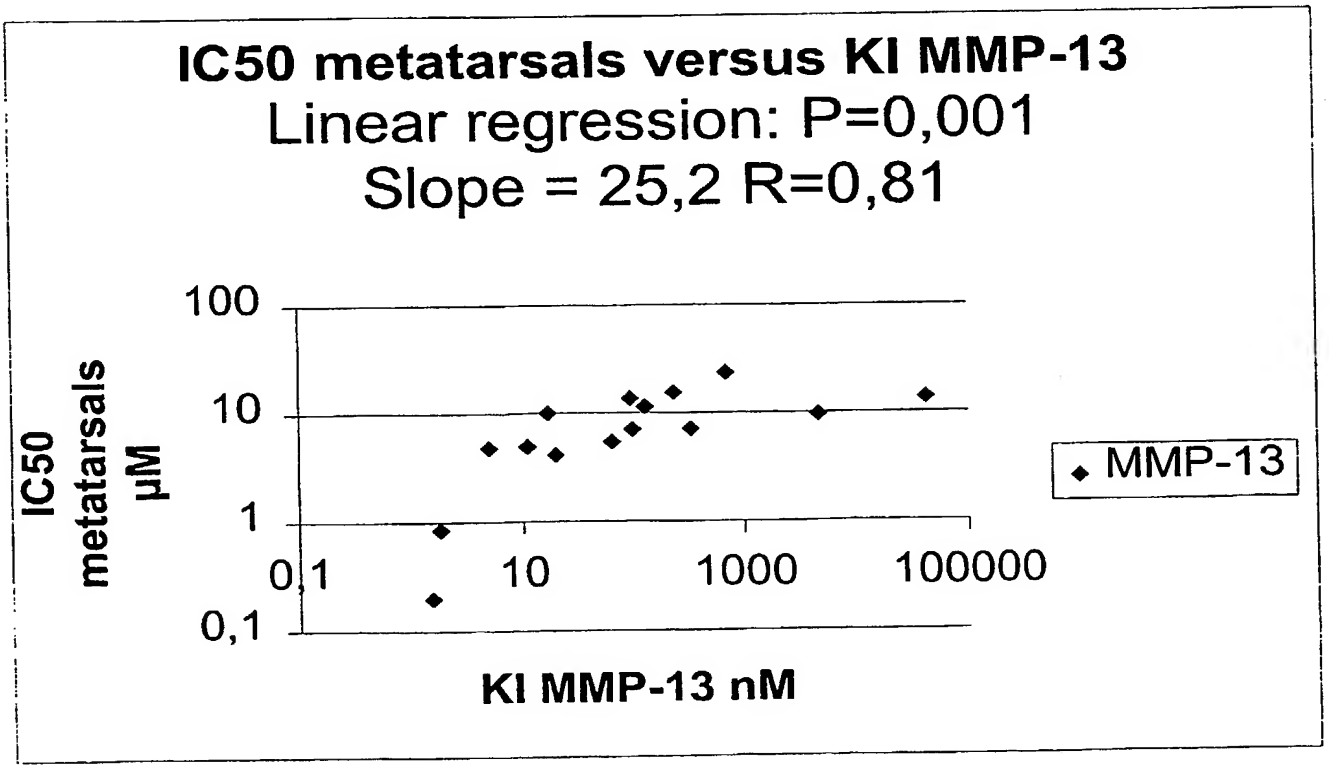

IC50 metatarsals versus KI MMP-13
Linear regression: P=0,001
Slope = 25,2 R=0,81
100
10
1
0,1
IC50 metatarsals µM
0,1
10
1000
100000
MMP-13
KI MMP-13 nM

Fig. 20

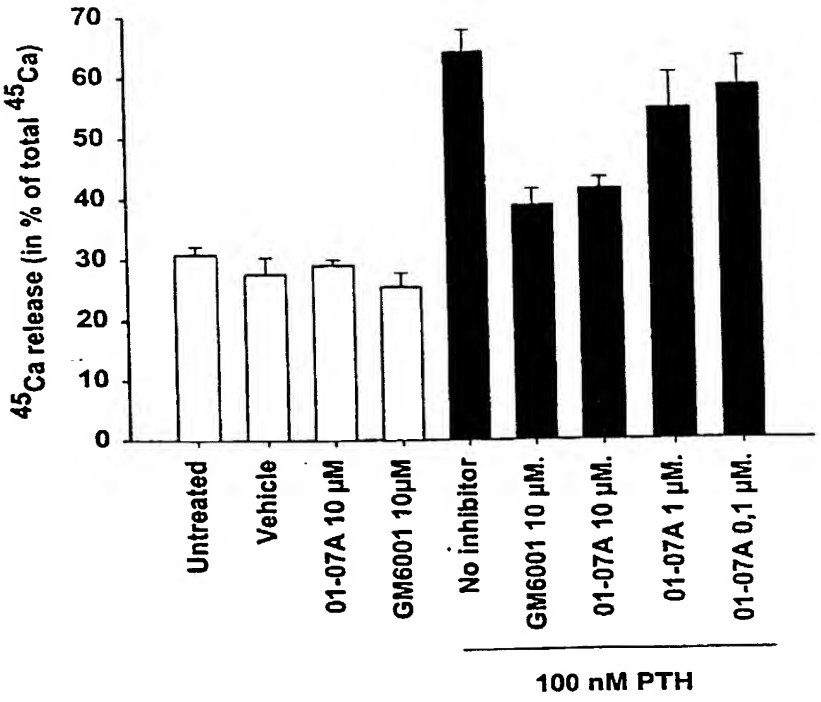

^{45}Ca release (in % of total ^{45}Ca)
70
60
50
40
30
20
10
0
Untreated
Vehicle
01-07A 10 µM
GM6001 10µM
No inhibitor
GM6001 10 µM.
01-07A 10 µM.
01-07A 1 µM.
01-07A 0,1 µM.
100 nM PTH

Fig. 21

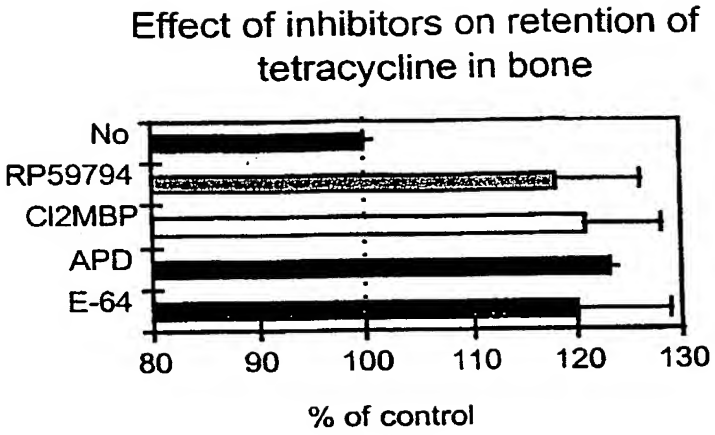

Effect of inhibitors on retention of tetracycline in bone
No
RP59794
Cl2MBP
APD
E-64
80
90
100
110
120
130
% of control

Fig. 22

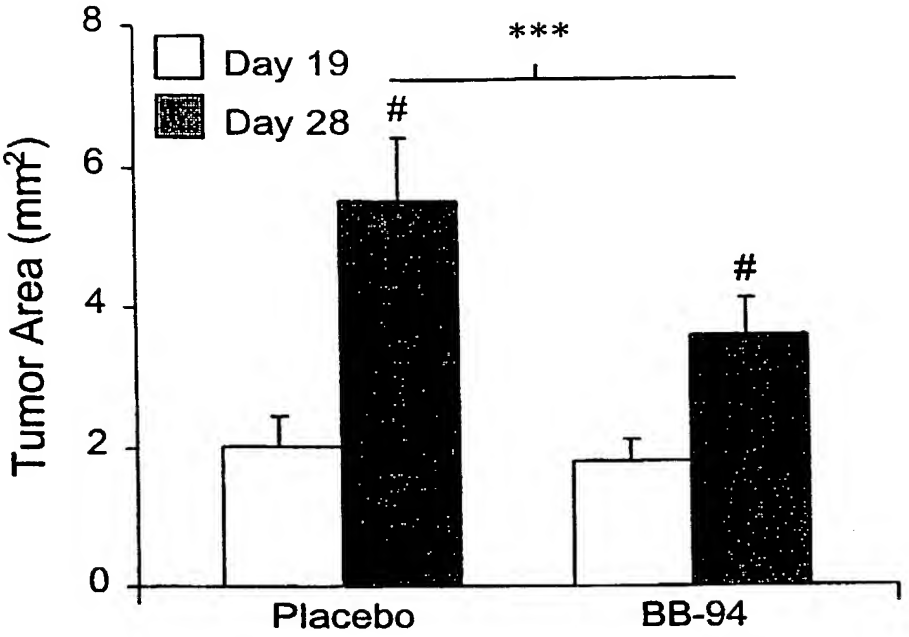


Fig. 23

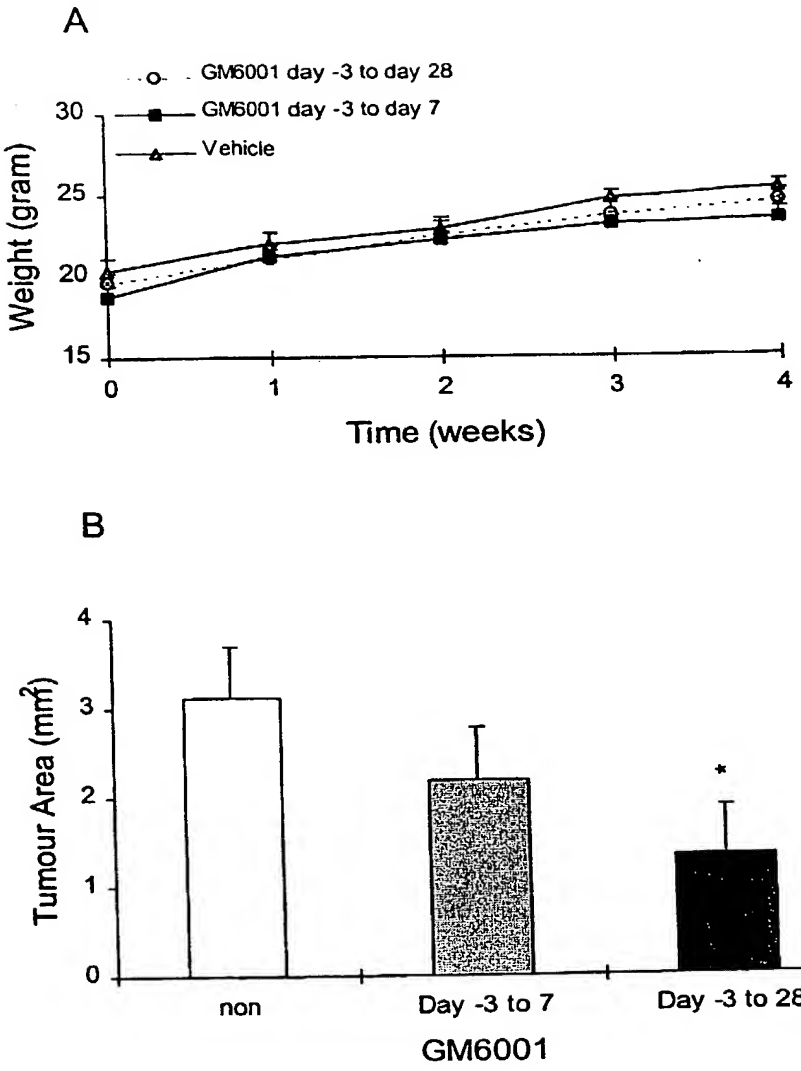

A
GM6001 day -3 to day 28
GM6001 day -3 to day 7
Vehicle
Weight (gram)
30
25
20
15
0
1
2
3
4
Time (weeks)
B
Tumour Area (mm²)
4
3
2
1
0
*
non
Day -3 to 7
Day -3 to 28
GM6001

Fig. 13

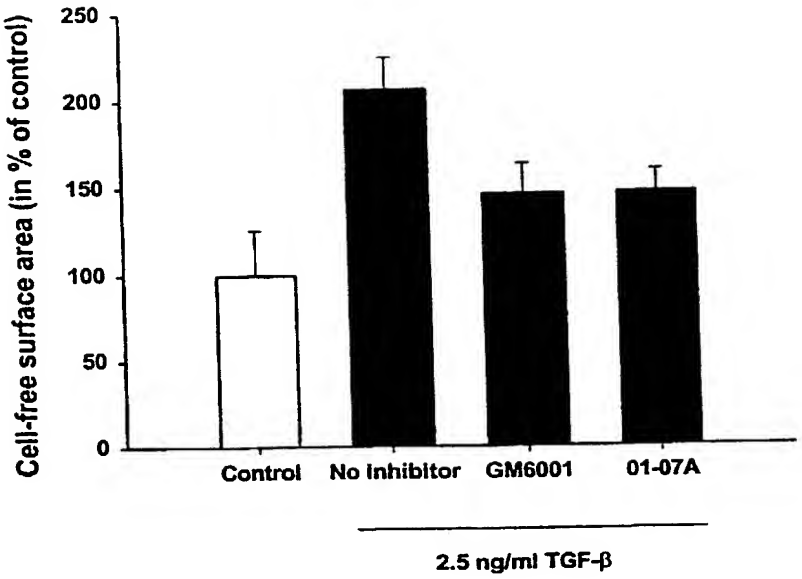

Cell-free surface area (in % of control)
250
200
150
100
50
0
Control
No inhibitor
GM6001
01-07A
2.5 ng/ml TGF-β

Fig. 14

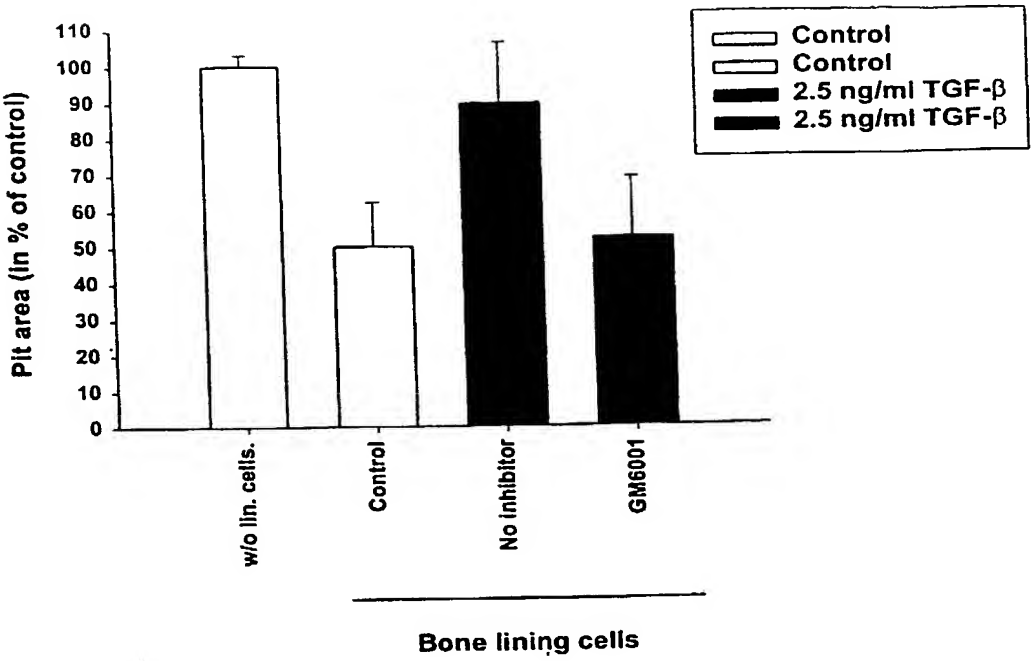

Control
Control
2.5 ng/ml TGF-β
2.5 ng/ml TGF-β
Pit area (in % of control)
110
100
90
80
70
60
50
40
30
20
10
0
w/o lin. cells.
Control
No inhibitor
GM6001
Bone lining cells

Fig. 15

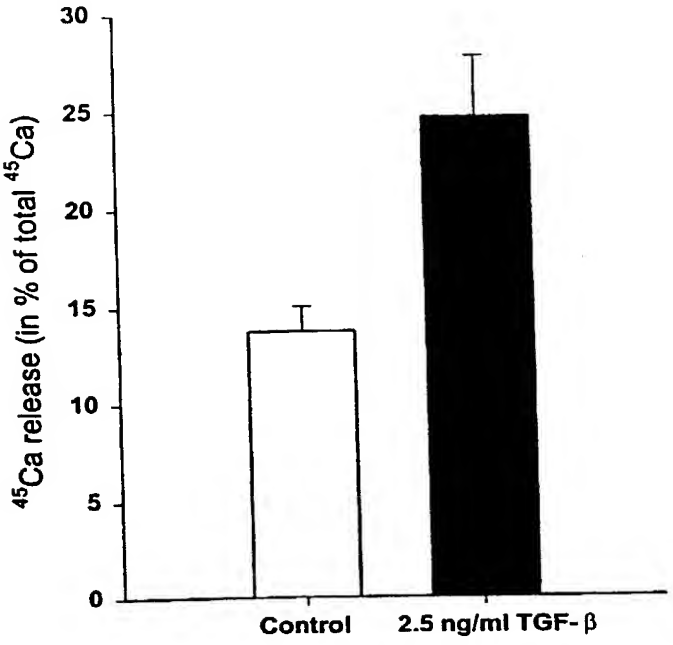

30
25
20
15
10
5
0
^{45}Ca release (in % of total ^{45}Ca)
Control
2.5 ng/ml TGF-β

Fig. 16

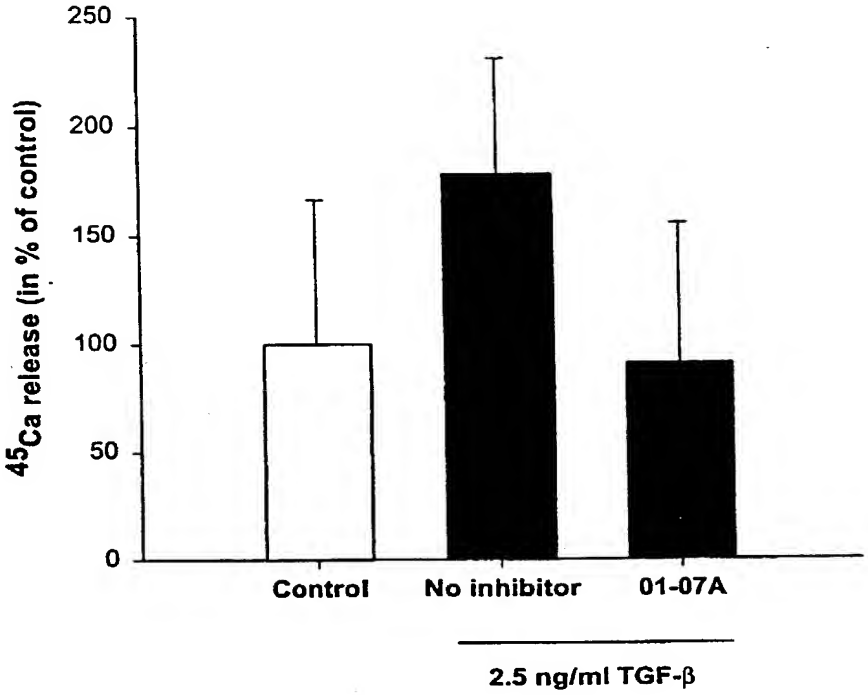

250
200
150
100
50
0
^{45}Ca release (in % of control)
Control
No inhibitor
01-07A
2.5 ng/ml TGF-β

Fig. 17

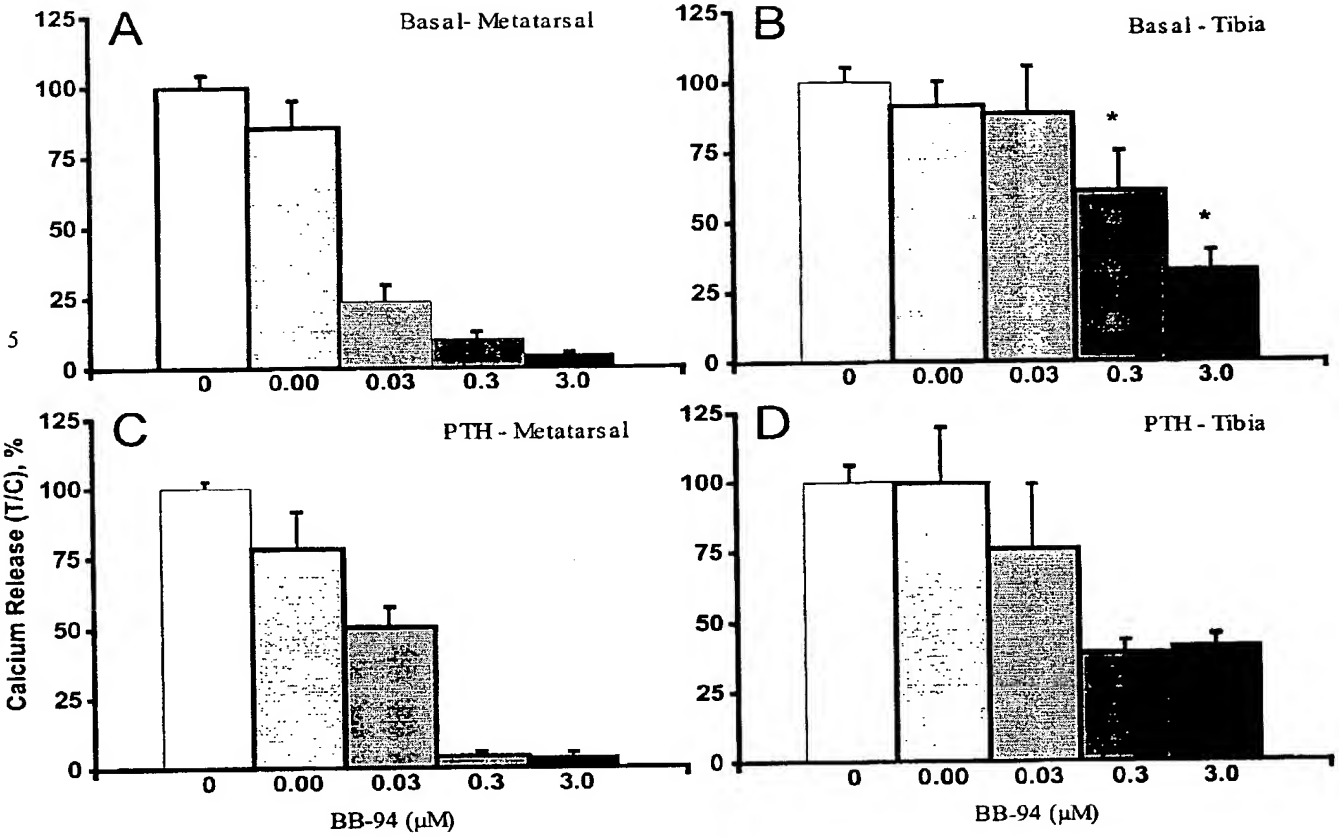

A
Basal- Metatarsal
B
Basal - Tibia
C
PTH - Metatarsal
D
PTH - Tibia
Calcium Release (T/C), %
125
100
75
50
25
0
0
0.00
0.03
0.3
3.0
*
BB-94 (μM)

Fig. 18

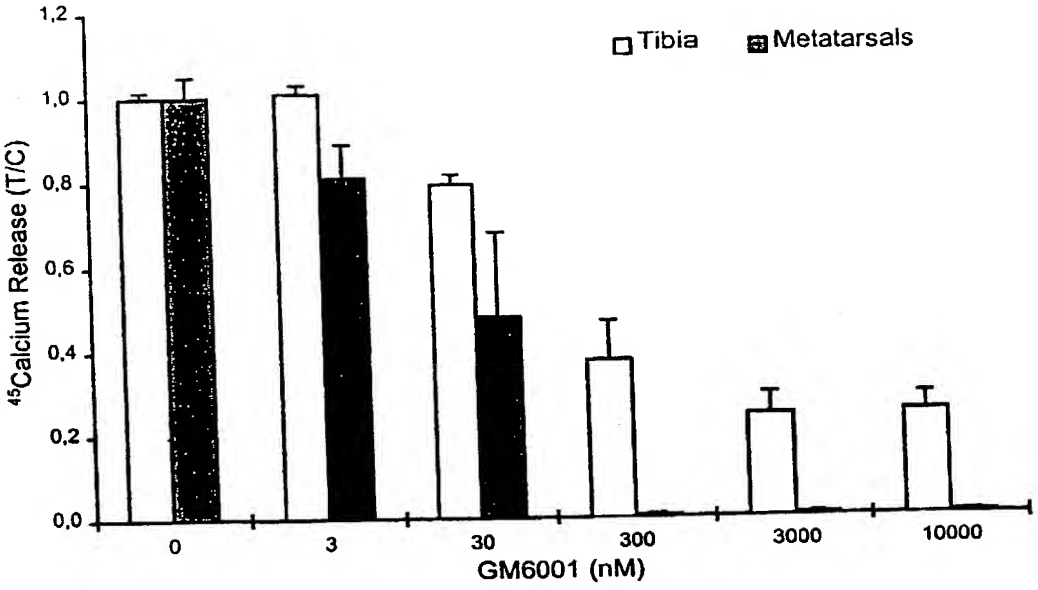

Tibia
Metatarsals
^{45}Calcium Release (T/C)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
3
30
300
3000
10000
GM6001 (nM)

Fig. 25

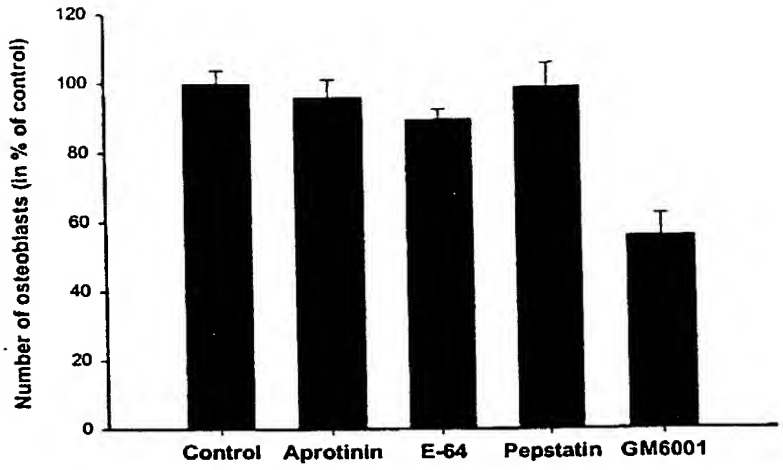

Number of osteoblasts (in % of control)
120
100
80
60
40
20
0
Control
Aprotinin
E-64
Pepstatin
GM6001

Fig. 26

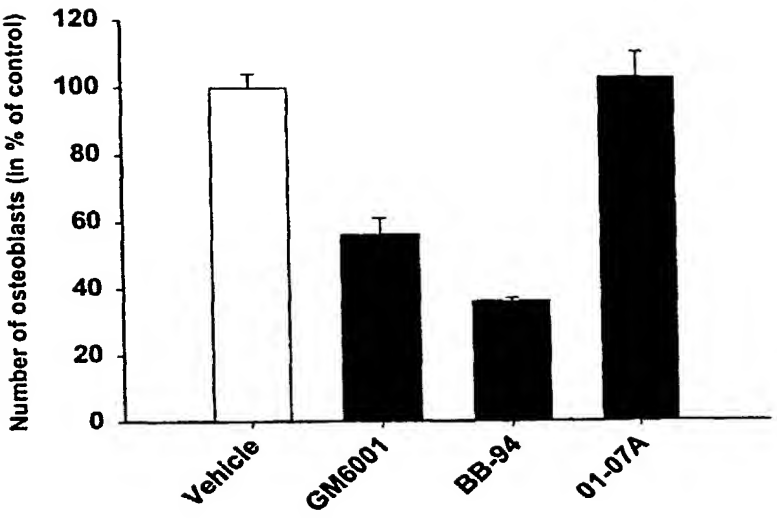

Number of osteoblasts (in % of control)
120
100
80
60
40
20
0
Vehicle
GM6001
BB-94
01-07A

Fig 27

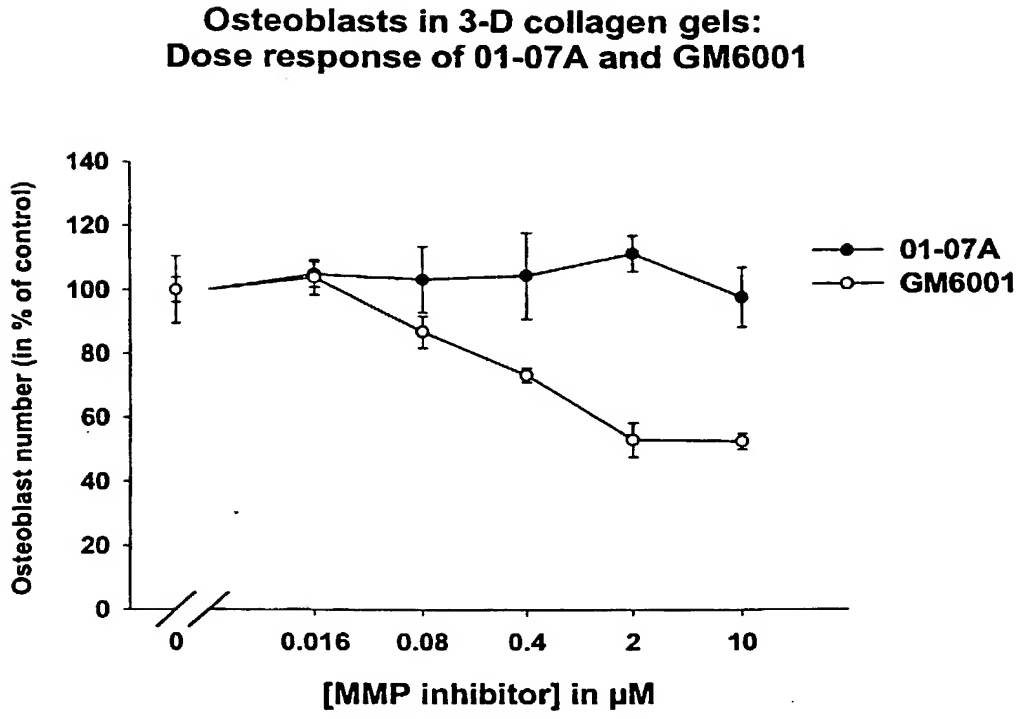

Osteoblasts in 3-D collagen gels:
Dose response of 01-07A and GM6001
Osteoblast number (in % of control)
140
120
100
80
60
40
20
0
0
0.016
0.08
0.4
2
10
[MMP inhibitor] in µM
01-07A
GM6001

Fig 28

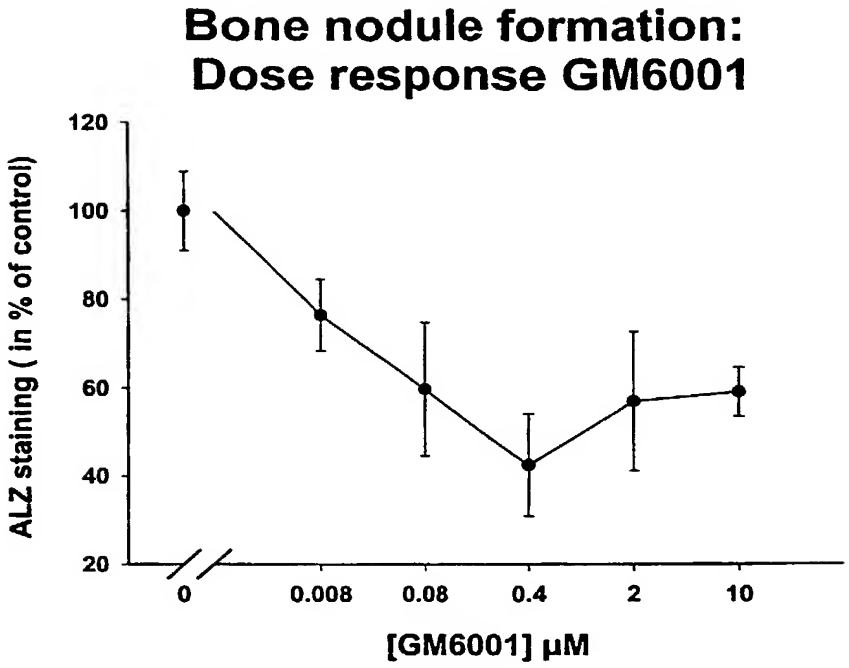
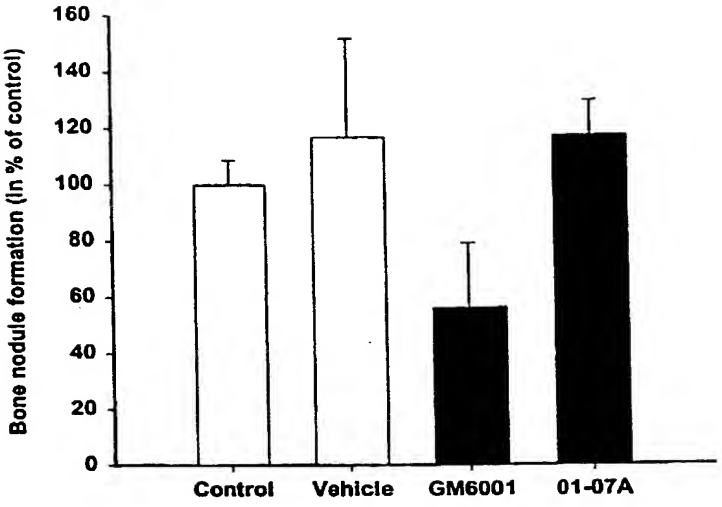

Bone nodule formation:
Dose response GM6001
ALZ staining (in % of control)
120
100
80
60
40
20
0
0.008
0.08
0.4
2
10
[GM6001] µM

Fig. 29


Bone nodule formation (in % of control)
160
140
120
100
80
60
40
20
0
Control
Vehicle
GM6001
01-07A

10083571.082202
PENNIE & EDMONDS LLP DOCKET NO.

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES

and for which a patent application:
☐ is attached hereto and includes amendment(s) filed on _____ *(if applicable)*
☐ was filed in the United States on _____ as Application No. _____ *(for declaration not accompanying application)*
with amendment(s) filed on _____ *(if applicable)*
☒ was filed as PCT international Application No. PCT/EP00/09173 on 19 September 2000 and was amended under PCT Article 19 on _____ *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
9922577.3	GB	23 September 1999	YES ☒ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), Gerald J. Flintoft (Reg. No. 20823), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Barry D. Rein (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Isaac Jarkovsky (Reg. No. 22713), Joseph V. Colaianni (Reg. No. 20019), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469), John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebel (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Jon R. Stark (Reg. No. 30111), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Samuel B. Abrams (Reg. No. 30605), Steven I. Wallach (Reg. No. 35402), Marcia H. Sundeen (Reg. No. 30893), Paul J. Zegger (Reg. No. 33821), Edmond R. Bannon (Reg. No. 32110), Bruce J. Barker (Reg. No. 33291), Adriane M. Antler (Reg. No. 32605), Ann L. Gisolfi (Reg. No. 31956), SaraLynn Mandel (Reg. No. 31853), Mark A. Farley (Reg. No. 33170), and James G. Markey (Reg. No. 31636), all of Pennie & Edmonds LLP, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 3300 Hillview Avenue, Palo Alto, CA 94304, and each of them, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

PEDC-98567.1

10088571.092202
PENNIE & EDMONDS LLP DOCKET NO.

SEND CORRESPONDENCE TO:	PENNIE & EDMONDS LLP 1667 K STREET, N.W. WASHINGTON, D.C. 20006			**DIRECT TELEPHONE CALLS TO:** PENNIE & EDMONDS LLP DOCKETING (202) 496-4400	
201	FULL NAME OF INVENTOR	LAST NAME BUCHARDT	FIRST NAME Jens	MIDDLE NAME -	
	RESIDENCE & CITIZENSHIP	CITY Copenhagen	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET Farmagsgade 69, 2th	CITY Copenhagen	STATE OR COUNTRY Denmark	ZIP CODE DK-1364
202	FULL NAME OF INVENTOR	LAST NAME FOGED	FIRST NAME Niels	MIDDLE NAME T.	
	RESIDENCE & CITIZENSHIP	CITY Veksø	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET Damvadvej 30, Søsum	CITY Veksø	STATE OR COUNTRY Denmark	ZIP CODE DK-3670
203	FULL NAME OF INVENTOR	LAST NAME MELDAL	FIRST NAME Morten	MIDDLE NAME -	
	RESIDENCE & CITIZENSHIP	CITY Frederiksberg	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET Nitivej 11	CITY Frederiksberg	STATE OR COUNTRY Denmark	ZIP CODE DK-2000
204	FULL NAME OF INVENTOR	LAST NAME DELAISSE	FIRST NAME Jean-Marie	MIDDLE NAME -	
	RESIDENCE & CITIZENSHIP	CITY Ballerup	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Belgium	
	POST OFFICE ADDRESS	STREET Egebjerg Bygade 235, 1th	CITY Ballerup	STATE OR COUNTRY Denmark	ZIP CODE DK-2750
205	FULL NAME OF INVENTOR	LAST NAME ENGSIG	FIRST NAME Michael	MIDDLE NAME - THYRRING	
	RESIDENCE & CITIZENSHIP	CITY ~~Vanlose~~ LYNGBY	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET STENGÅRDS ALLE 8 ~~Hogholtvej 34~~	CITY ~~Vanlose~~ LYNGBY	STATE OR COUNTRY Denmark	ZIP CODE 2800 DK-~~2720~~
206	FULL NAME OF INVENTOR	LAST NAME FERRERAS	FIRST NAME Mercedes	MIDDLE NAME -	
	RESIDENCE & CITIZENSHIP	CITY Copenhagen	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Spain	
	POST OFFICE ADDRESS	STREET Jagtvej 201	CITY Copenhagen	STATE OR COUNTRY Denmark	ZIP CODE DK-2100

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 ~~25/3-20~~ Jens Buchardt	SIGNATURE OF INVENTOR 202 Niels Foged	SIGNATURE OF INVENTOR 203 Morten Meldal
DATE 25/3-2002	DATE 8/5-2002	DATE 4/4-2002
SIGNATURE OF INVENTOR 204 J Delaisse	SIGNATURE OF INVENTOR 205 Michael Engsig	SIGNATURE OF INVENTOR 206 M. Ferreras
DATE 7/5/2002	DATE 8/5-2002	DATE 05-April-2002

10088571.082202

PENNIE & EDMONDS LLP DOCKET NO.

SEND CORRESPONDENCE TO: PENNIE & EDMONDS LLP
1667 K STREET, N.W.
WASHINGTON, D.C. 20006

DIRECT TELEPHONE CALLS TO:
PENNIE & EDMONDS LLP DOCKETING
(202) 496-4400

207	FULL NAME OF INVENTOR	LAST NAME ~~KARSDAL~~	FIRST NAME Morten	MIDDLE NAME - ASSER	
	RESIDENCE & CITIZENSHIP	CITY Copenhagen	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET Ndr. Frihavnsgade 100A	CITY Copenhagen	STATE OR COUNTRY Denmark	ZIP CODE DK-2100
208	FULL NAME OF INVENTOR	LAST NAME OVEJERO	FIRST NAME Maria del Carmen	MIDDLE NAME -	
	RESIDENCE & CITIZENSHIP	CITY Copenhagen	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Spain	
	POST OFFICE ADDRESS	STREET Østerbrogade 118	CITY Copenhagen	STATE OR COUNTRY Denmark	ZIP CODE DK-2100
209	FULL NAME OF INVENTOR	LAST NAME SCHIØDT	FIRST NAME Christine	MIDDLE NAME B.	
	RESIDENCE & CITIZENSHIP	CITY Brønshøj	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET Risbyholmvej 20	CITY Brønshøj	STATE OR COUNTRY Denmark	ZIP CODE DK-2700
210	FULL NAME OF INVENTOR	LAST NAME WINDING	FIRST NAME Bent	MIDDLE NAME -	
	RESIDENCE & CITIZENSHIP	CITY ~~Copenhagen~~ Hørsholm	STATE OR FOREIGN COUNTRY Denmark DK	COUNTRY OF CITIZENSHIP Denmark	
	POST OFFICE ADDRESS	STREET ~~Bartolinsgade 1~~ BLOMSTRØDVEJ 26B	CITY ~~Copenhagen~~ HØRSHOLM	STATE OR COUNTRY Denmark	ZIP CODE 2970 ~~DK-1356~~
211	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
212	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 207	SIGNATURE OF INVENTOR 208	SIGNATURE OF INVENTOR 209
DATE 4/5-2002	DATE 13/5-2002	DATE May 8th 2002
SIGNATURE OF INVENTOR 210	SIGNATURE OF INVENTOR 211	SIGNATURE OF INVENTOR 212
DATE 15/5-2002	DATE	DATE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (c)) - SMALL BUSINESS CONCERN	Docket No. 46865/57841 (8969-033-999)

Serial No.	Filing Date	Patent No.	Issue Date
10/088,571	March 21, 2002		

Applicant/ Patentee: Jens BUCHARDT, ET AL.

Invention: SUBSTITUTED PHOSPHINATE BASED PEPTIDE DERIVATIVES

I hereby declare that I am:

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: **Nordic Bioscience A/S**
ADDRESS OF CONCERN: **Herlev Hovedgade 207, DK-2730 Herlev, Denmark**

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above identified invention described in:

- ☐ the specification filed herewith with title as listed above.
- ☒ the application identified above.
- ☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☐ no such person, concern or organization exists.
- ☐ each such person, concern or organization is listed below.

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Klaus Eldrup-Jørgensen

TITLE OF PERSON SIGNING OTHER THAN OWNER: CEO

ADDRESS OF PERSON SIGNING: **Nordic Bioscience A/S**
Herlev Hovedgade 207
DK-2730 Herlev, Denmark

SIGNATURE: _____ DATE: Sept. 11, 2002